The Proprotein Convertase Furin is a Key Regulator of Paediatric Sarcoma Malignancy

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To my mother

Who is not only a survivor but lives life to the fullest

"Logic will get you from A to Z; imagination will get you everywhere."

Albert Einstein

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Summary

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and adolescents. Paediatric RMS is classified into two histologically and molecularly distinct subtypes: embryonal RMS (eRMS) and alveolar RMS (aRMS). The more common eRMS subtype is present in 60-70% of all cases and is associated with a 5-year overall survival rate of over 60%, whereas aRMS, occurring in 20-30% of all cases, has a dismal 5-year overall survival rate of 22%. Furthermore, the prognosis for eRMS and aRMS patients presenting with metastatic disease is particularly poor. Multimodal therapy plans encompass chemotherapy, surgery and radiotherapy and often lead to acute and chronic morbidities. Hence, novel, complementing treatment options have to be established in order to improve treatment outcome and reduce the risk of short and long term side effects.

In this project, we identified and characterised the proprotein convertase (PC) furin as important regulator of RMS maintenance and progression. In an initial study we analysed the expression pattern of PCs in paediatric RMS, Ewing sarcoma and osteosarcoma cell lines and found that their expression was particularly high in RMS cells, whereby furin showed the most consistent expression throughout all investigated cell lines.

To investigate the role of furin in important cellular processes *in vitro* and *in vivo* we employed different approaches to modulate furin activity. RMS cell lines with enhanced furin activity were generated through stable ectopic expression of furin. To reduce furin activity on the other hand, we used different means: 1) application of decanoyl-RVKR-chloromethylketone (CMK), a pan-PC inhibitor; 2) stable expression of the pan-PC inhibitor PDX; 3) RNA interference through transfection of siRNA or tetracycline-inducible expression of shRNA.

Analysis of Rh30 (aRMS) and RD (eRMS) cells with stable modulation of furin activity showed that reduced furin activity delays RMS tumour growth *in vivo*, most likely through impaired vessel formation in the initial tumour growth phase. Furthermore, we found evidence that furin plays a role in metastatic processes, as a decrease of furin activity resulted in reduced migratory and invasive behaviour of RMS cells *in vitro*. To elucidate the underlying mechanisms, we assessed the processing of known furin substrates that have been previously associated with RMS progression and metastasis and observed impaired IGF1R, VEGF-C, PDGF-BB and MT1-MMP maturation upon application of the inhibitor CMK. Additionally, we could confirm that the proteolytic activation of IGF1R through furin is necessary in order to elicit IGF1R signalling in RMS cells upon stimulation with IGF-1.

Many of the known furin substrates are associated with proliferation and survival of cancer cells. IGF signalling, for instance, plays an important role in RMS maintenance.

Therefore, we sought to further characterise the implication of furin activity in proliferation and survival of RMS cells. For this purpose, we established various aRMS cell lines with tetracycline-inducible furin shRNA expression to study the immediate effects of furin silencing, in a way mimicking the effect of a very specific and effective inhibitor. We found that silencing of furin impaired cell viability and proliferation in all investigated aRMS cell lines, but had no effect in the fibroblast cell line MRC5. Furthermore, half of the aRMS cell lines investigated, including Rh3 and Rh4 cells, seemed to be more sensitive to furin depletion, as loss of furin activity further elicited induction of apoptosis. Subsequent analysis of apoptosis showed that the process is dependent on caspase activity and most likely involves pore formation in mitochondria, as a double knock-out of BAX and BAK abolished furin silencing induced cell death. A similar pattern of sensitivity could also be confirmed *in vivo*, as furin silencing in the sensitive Rh4 cells led to complete regression of tumours, while reduced furin activity in Rh30 xenografts resulted in delayed tumour growth. Apoptosis upon depletion of furin in Rh4 xenografts was confirmed by the presence of cleaved caspases and PARP as analysed by immunoblot and immunohistochemistry.

In addition, we assessed the expression pattern of furin protein in a total of 89 eRMS and aRMS biopsies on a tissue microarray and could confirm furin expression in 86% of the tumours. In conclusion, our findings identify and validate furin as an important factor for RMS progression, survival and metastasis and we therefore propose furin as a novel therapeutic target for treatment of paediatric RMS. Thus, furin-specific inhibitors could be developed as their use, possibly in combination with inhibitors targeting other key signalling molecules like IGF1R, represents a promising approach for complementing established chemotherapeutic regimens. Furthermore, as we could show previously, furin is a homing receptor for RMS-specific peptides and might accordingly be exploited for targeted delivery of chemotherapeutics, thereby reducing some of the side effects of conventional chemotherapy.

Zusammenfassung

Rhabdomyosarkome (RMS) sind die am häufigsten vorkommenden Weichteilsarkome bei Kindern und Jugendlichen. Zwei molekulare Haupttypen pädiatrischer RMS werden voneinander unterschieden: embryonale RMS (eRMS) und alveoläre RMS (aRMS). 60-70% aller RMS-Fälle gehen auf den häufiger auftretenden Subtyp eRMS zurück, der mit einer 5-Jahresüberlebensrate von über 60% assoziiert ist. aRMS hingegen kommt seltener vor und entspricht 20-30% aller Fälle. Im Gegensatz zu eRMS ist die Prognose für aRMS relativ schlecht: die 5-Jahresüberlebensrate beträgt lediglich 22%. Die geringste Überlebenschance haben Patienten mit metastasierenden eRMS und aRMS Primärtumoren. Multimodale Therapieansätze umfassen Chemo- und Strahlentherapie sowie, soweit möglich, eine chirurgische Entfernung des Tumors. Leider führen eben diese Therapien in vielen Fällen zu sekundären Erkrankungen. Daher könnten neue, komplementierende Therapiemöglichkeiten nicht nur Behandlungserfolge verbessern, sondern gleichzeitig auch das Risiko für Nebenwirkungen und Spätfolgen verringern.

In dieser Arbeit wurde die Proproteinkonvertase Furin als wichtiger Faktor zur Erhaltung und Progression von RMS-Zellen identifiziert und *in vitro* und *in vivo* charakterisiert. Zunächst haben wir das Expressionsmuster von allen Proproteinkonvertasen in pädiatrischen Sarkom-Zelllinien analysiert und festgestellt, dass diese besonders stark in RMS-Zelllinien, nicht aber in Ewingsarkom- und Osteosarkom-Zelllinien exprimiert werden. Furin war dabei die Proproteinkonvertase mit den gleichmässig höchsten Levels.

Zur Untersuchung der Rolle von Furin in RMS kamen unterschiedliche Ansätze zur Modulierung der Furinaktivität zum Einsatz. Stabil erhöhte Furinaktivität wurde durch ektopische Furin-Expression erzielt und unterschiedliche Ansätze konnten etabliert werden um die Furinaktivität zu reduzieren: 1) Verwendung des pan-Proprotein Konvertase Inhibitors Dekanoyl-RVRK-Chloromethylketon (CMK); 2) Stabile Expression des pan-Proprotein Konvertase Inhibitors PDX; 3) RNA-Interferenz durch Transfektion von siRNA oder durch Tetracyclin-induzierbare Expression von shRNA.

Durch den Einsatz stabiler Zelllinien mit modulierter Furinaktivität fanden wir heraus, dass eine niedrige Furinaktivität den Wachstum von RMS-Tumoren *in vivo* verzögert, was mit hoher Wahrscheinlichkeit auf unzureichende Blutgefässbildung in frühen Stadien des Tumorwachstums zurückzuführen ist. Des Weiteren spielt Furin eine Rolle für die Metastasierung von RMS-Zellen: eine geringere Furinaktivität beeinträchtigt das migratorische und invasive Verhalten von Rh30 (aRMS) und RD (eRMS) Zellen *in vitro*. Um die zugrundeliegenden Mechanismen aufzuklären haben wir die proteolytische Prozessierung von bekannten Furin-Substraten untersucht. Eine Inhibierung von Proproteinkonvertasen durch

den Inhibitor CMK führt zu einer stark verringerten Prozessierung und Aktivierung der Furin-Substrate IGF1R, VEGF-C, PDGF-BB und MT1-MMP. Zusätzlich konnten wir zeigen, dass die proteolytische Aktivierung von IGF1R notwendig ist zur Stimulierung der in RMS-Zellen wichtigen IGF-Signalkaskade.

Viele der bekannten Furin-Substrate sind mit dem Überleben und der Proliferation von Krebszellen assoziiert worden. Der IGF-Signalweg zum Beispiel spielt eine zentrale Rolle für den Erhalt von RMS-Zellen. Aus diesem Grund haben wir die Bedeutung von Furin für die Proliferation und das Überleben von RMS-Zellen genauer untersucht. Hierfür wurden unterschiedliche aRMS Zelllinien mit Tetracyclin-induzierbarer Furin shRNA generiert um die unmittelbaren Effekte einer Furin-Eliminierung, wie es beim Einsatz eines sehr spezifischen und potenten Inhibitors der Fall wäre, zu analysieren. Ein Verlust der Furinaktivität beeinträchtigte stark die Proliferation aller untersuchter aRMS Zelllinien, hatte jedoch keinen Einfluss auf die Wachstumsrate von MRC5 Fibroblasten. In der Hälfte aller aRMS Zelllinien, einschliesslich Rh3 und Rh4, führte ein Verlust von Furin zum Zelltod durch Apoptose. Anschliessende detaillierte Experimente zeigten, dass der Prozess im Zusammenhang mit Caspase-Aktivität und BAX/BAK-abhängiger mitochondrialer Porenbildung steht. Der Unterschied zwischen den Zelllinien zeigte sich auch *in vivo*, wo reduzierte Furinaktivität zur kompletten Tumorregression der sehr sensitiven Rh4-Zellen führte, wohingegen Rh30-Tumore lediglich langsamer wuchsen. Apoptose nach Verlust von Furinaktivität in Rh4-Tumoren wurde durch Anwesenheit von geschnittenen Caspasen und PARP per Westernblot und Immunhistochemie nachgewiesen.

Darüber hinausgehend konnten wir anhand eines Tumor-Microarrays der 89 Biopsien umfasst die Expression von Furin-Protein in 86% der RMS-Tumore bestätigen. Somit validieren unsere Studien Furin als wichtigen Faktor für den Erhalt, das Überleben und die Metastasierung von RMS-Zellen und zeigen die Möglichkeit einer Furin-spezifischen neuen Behandlungsmöglichkeit von RMS auf. Aufbauend auf dieser Studie könnten zukünftig Furin-spezifische Inhibitoren zum Einsatz kommen, möglicherweise in Kombination mit Inhibitoren für weitere validierte Signalmoleküle wie IGF1R. Alternativ kann Furin, wie wir in früheren Studien zeigen konnten, als Zielrezeptor für RMS-spezifische Peptide dienen um Chemotherapeutika gezielter in den Tumor zu transportieren. Dadurch können die Belastung für den Patienten und das Risiko für Chemotherapeutika-assoziierte Nebenwirkungen potentiell gesenkt werden.

List of abbreviations

α1-PDX	α1-antitrypsine Portland
αMSH	α-melanocyte-stimulating hormone
Aβ	amyloid beta
ACCIS	European Automated Childhood Cancer Information System
ACTH	adrenocorticotropic hormone
ADAM	a disintegrin and metalloprotease
ADAM-TS	a disintegrin and metalloprotease with thrombospondin motif
ADH	autosomal dominant hypercholersterolaemia
AP	adaptor protein
APP	amyloid precursor protein
aRMS	alveolar rhabdomyosarcoma
ATF6	activating transcription factor 6
BACE	β-site amyloid precursor protein cleaving enzyme
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
CAM	cell adhesion molecule
CCHF Gn	Crimean–Congo haemorrhagic fever virus glycoprotein Gn
COG	Children's Oncology Group
CHRD	Cys-His-rich domain
CMK	Decanoyl-RVKR-chloromethylketone
CREB	cyclic AMP-responsive element binding protein
CRD	Cys-rich domain
ECM	extracellular matrix
EF	edema factor
ER	endoplasmatic reticulum
eRMS	embryonal rhabdomyosarcoma
FAL	fractional allelic loss
FGFR	fibroblast growth factor receptor
FRET	Förster resonance energy transfer
GCCR	German Childhood Cancer Registry
GDF11	growth differentiation factor 11
GHRH	growth hormone-releasing hormone
GlcNAc	N-acetylglucosamine
GLP1	glucagon-like peptide 1

GM-CSF	granulocyte macrophage colony-stimulating factor
gp	envelope glycoprotein
HGF	hepatocyte growth factor
HIV	human immunodeficiency virus
HNSCC	head and neck squamous cell carcinoma
HSPG	heparan sulphate proteoglycans
ICCC	international classification of childhood cancer
IGF	insulin-like growth factor
IGF1R	insulin-like growth factor 1 receptor
IRSG	Intergroup Rhabdomyosarcoma Study Group
L1CAM	L1 cell adhesion molecule
LF	lethal factor
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LOH	loss of heterozygosity
LOI	loss of imprinting
LRP8	LDLR-related protein 8
MCH	melanin concentrating hormone
MMP	matrix metalloprotease
MT-MMP	membrane-type matrix metalloprotease
NGF	nerve growth factor
NMR	nuclear magnetic resonance
NSCLC	non-small cell lung carcinoma
OS	overall survival
PA	protective antigen
pa83	protective antigen 83
PACAP	pituitary adenylyl cyclase-activating peptide
PACE4	paired basic amino acid cleaving enzyme 4
PACS-1	phosphofurin acidic cluster sorting protein-1
PC	proprotein convertase
PCSK9	proprotein convertase subtilisin kexin 9
PDGF	platelet derived growth factor
PDGFRβ	platelet derived growth factor receptor β
PDX	α1 antitrypsin Portland
pK_a	acid dissociation constant
POMC	pro-opiomelanocortin

PP2A	protein phosphatase 2A
PTH	parathyroid hormone
PTPRM	protein tyrosine phosphatase receptor type M
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RGMA	repulsive guidance molecule A
RMS	rhabdomyosarcoma
S1P	site-1 protease
SCLC	small cell lung carcinoma
SEER	Surveillance, Epidemiology, and End Results program
serpin	serine protease inhibitor
SKI-1	subtilisin kexin isozyme 1
SREBP	sterol regulatory element-binding protein
TACE	tumour necrosis factor α convertase
TGFβ	transforming growth factor-β
TGN	trans-Golgi network
TIMP	tissue inhibitor of metalloproteases
TRH	thyrotropin releasing hormone
uPA	urokinase-type plasminogen activator
VAC	vincristine, actinomycin-d and cyclophosphamide
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VLDLR	very-low-density lipoprotein receptor
WHO	world health organisation

Chapter 1

Introduction

1. Cancer in adults and children

Cancer represents one of the leading causes of mortality worldwide with an estimated 8.2 million cancer related deaths in 2012 (Stewart and Wild 2014). Cancers may appear anywhere in the body and the World Health Organisation (WHO) estimated that over 100 individual cancer types exist, however cancers in the lung, the liver, the stomach, the breast and colorectal cancers are the most common and account together for more than half of all deaths (Stewart and Wild 2014). The development of a normal cell into a tumour cell is a multistage process, involving several transformation steps and thus the incidence for most cancer types rises immensely with age. Besides genetic predisposition, several external factors have been described to increase the risk of cancer development; these include tobacco and alcohol use, unhealthy diet and physical inactivity as well as viral infections such as infection with hepatitis B and C viruses or human papilloma virus (WHO 2015).

Paediatric cancers are cancers that occur in children under the age of 14, however, depending on the definition, cancers in adolescents (age 14-19) are often included. It is estimated that over 175'000 children develop cancer every year and despite their rarity childhood cancers represent the leading cause of death in developed countries with more than 96'000 deaths in 2008 (American Cancer Society 2011). Nevertheless, mortality rates have clearly decreased in developed countries since the 1970s, most likely due to widely improved treatment modalities (Yang, Fujimoto et al. 2009; Bosetti, Bertuccio et al. 2010). The overall incidence rate of paediatric cancer has risen slightly over the last decades, for instance from 11.5 per 100'000 in 1975 to 14.3 per 100'000 in 2007 in the US, which might in part be attributed to improved diagnosis and reporting procedures, but true increases in incidence have been observed for some types such as leukaemias and soft tissue sarcomas (Kaatsch, Steliarova-Foucher et al. 2006; Shah and Coleman 2007). In general, trends for incidence and mortality of childhood cancers are more complicated to analyse in third world countries for example due to incomplete documentation of cases (Howard, Metzger et al. 2008). The most common cancer types observed in children are leukaemias at 34%, brain tumours at 23% and lymphomas at 12% (reviewed in (Kaatsch 2010).

2. Sarcomas

The term sarcoma is derived from the greek word σάρξ *sarx*, which means “flesh”. This type of cancer originates from cells of mesenchymal origin and may therefore develop in any kind of connective tissue. Sarcomas are grouped according to the cell type they are derived from and include, among others, fibrosarcomas (fibroblasts), osteosarcoma (osteoblasts), liposarcoma (adipocytes), angiosarcoma (precursor endothelial cells) and rhabdomyosarcoma (myoblasts) (Weinberg 2007).

The overall incidence for sarcomas across all age groups in Europe is 5.6 per 100’000 per year, accounting for roughly 28’000 new cases. 84% of all sarcomas are soft tissue sarcomas and 14% are sarcomas arising in the bone (Stiller, Trama et al. 2013). Whereas sarcomas are rather rare in adults, representing only about 1% of all cases, they are more common in children and adolescents and account for approximately 10% of all registered cancer cases (source: German Childhood Cancer Registry (GCCR); reviewed in (Kaatsch 2010)). The incidence rate of paediatric soft tissue sarcomas, as classified by the International Classification of Childhood Cancer (ICCC) diagnostic groups, is 9.1 per million children per year (source: European Automated Childhood Cancer Information System (ACCIS); reviewed in (Kaatsch 2010)). With the advent of chemotherapeutic modalities in the 1970s, prognosis for children diagnosed with sarcomas improved dramatically. Thus, the 5-year overall survival for paediatric soft tissue sarcomas reached 65% in Europe (source: ACCIS, 1988-1997 (Sankila, Martos Jimenez et al. 2006)) and 73% in the US (source: SEER (Surveillance, Epidemiology, and End Results program), 1985-1999 (Howlader, Noone et al. 2014)).

3. Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and adolescents and represents 55% of all registered soft tissue sarcomas and around 3% of all childhood malignancies (source: German Childhood Cancer registry; reviewed in (Kaatsch 2010)). The age-standardised incidence rate of paediatric RMS in Europe was 5.4 per million children in the 1993-1997 period (source: ACCIS (Pastore, Peris-Bonet et al. 2006)). Based on histology, childhood RMS can be grouped into two main subgroups: embryonal RMS (eRMS) and alveolar RMS (aRMS). eRMS is the most common subtype and represents around 66% of all cases. aRMS and botryoid RMS are less common, accounting for 22% and 11% of all cases, respectively (Van Gaal, Van Der Graaf et al. 2012).

The current 5-year overall survival rate of children with RMS, regardless of the subclassification, is estimated to be 70-80% (Crist, Anderson et al. 2001; Stevens, Rey et al. 2005; Dantonello, Int-Veen et al. 2009). Overall survival rates for botryoid, embryonal and alveolar RMS are around 78%, 64% and 22%, respectively (Figure 1A; (Van Gaal, Van Der Graaf et al. 2012)). Besides the histological subtype, presence of metastasis is one of the most important factors influencing prognosis of RMS patients. Thus, 5-year overall survival of patients presenting with non-metastatic eRMS or aRMS is 69% and 34%, respectively, whereas patients with metastasised disease have a very dismal prognosis of less than 12% (Figure 1B (Van Gaal, Van Der Graaf et al. 2012)). Adults suffering from RMS have an even worse prognosis than children, as the corresponding 5-year overall survival rate is as low as 27% (Sultan, Qaddoumi et al. 2009).

Risk stratification is based on a combination of the classical TNM staging system and the surgical/pathologic clinical grouping system as introduced by the Intergroup Rhabdomyosarcoma Study Group (IRSG) and includes various key factors (reviewed in (Malempati and Hawkins 2012)). These factors include, among others, adequacy of excision, subtype, age, stage of disease, primary site and presence of metastasis at time of diagnosis. For instance, it was observed that children ≥ 10 years as well as infants fared worse, the latter at least partly due to higher occurrence of local failure (Ferrari, Casanova et al. 2003; Joshi, Anderson et al. 2004; Malempati, Rodeberg et al. 2011).

One central point of diagnosis of RMS is the characterisation of embryogenic myogenesis. Such identification is mostly based on histologic assessment, ultrastructural examinations or immunohistochemical investigation of myogenic markers, including desmin, myosin, myoglobin, MyoD and myogenin (reviewed in (Parham and Barr 2013)). However, in order to distinguish RMS from other potentially myogenic neoplasms such as Wilms tumours, further histologic patterns characteristic for the distinct subtypes of RMS were defined on the

basis of the Horn-Enterline system, which was introduced in the late 1950s (Horn and Enterline 1958). Additionally, aRMS are most often characterised by the presence of non-random chromosomal translocations, giving rise to aberrant transcription factors, as will be discussed in section 3.1.

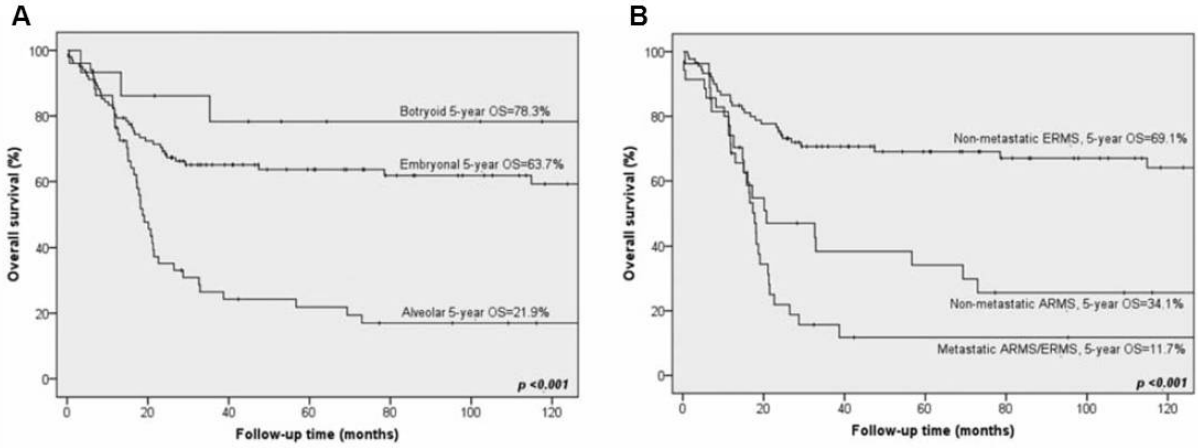

Figure 1 Overall survival of patients diagnosed with rhabdomyosarcoma.
Kaplan-Meier overall survival (OS) curves for patients with rhabdomyosarcoma (RMS) by histological subtype (A), botryoid RMS: N=15 patients, embryonal RMS: N=102 patients, and alveolar RMS: N=51 patients; and according to presence of metastasis at time of diagnosis in embryonal and alveolar RMS (B), non-metastatic embryonal RMS: N=90 patients, non-metastatic alveolar RMS: N=27 patients, metastatic embryonal and alveolar RMS: N=35 patients. ARMS, alveolar rhabdomyosarcoma; ERMS, embryonal rhabdomyosarcoma.
Adapted from. (Van Gaal, Van Der Graaf et al. 2012).

Furthermore, RMS, as other soft tissue sarcomas, appears to be more frequent in boys with a male to female ratio of 1.3-1.4 (Pastore, Peris-Bonet et al. 2006; Kaatsch 2010). However, this male predominance seems to be more prevalent in eRMS with a male to femal ratio of 1.51 (Ognjanovic, Linabery et al. 2009).

RMS may arise at many different sites throughout the body, whereby tumours appear most frequently in the head and neck region, in the genitourinary region, in the pelvis, in the trunk or in extremities (reviewed in (Pastore, Peris-Bonet et al. 2006)).

3.1. Embryonal and alveolar rhabdomyosarcoma

Embryonal RMS (eRMS) and alveolar RMS (aRMS) constitute the two most common paediatric RMS subtypes, but differ in many aspects, most importantly in their genetic background and key proteins that drive tumourigenesis and tumour progression.

eRMS occurs much more frequently in children than aRMS with an age-standardised incidence rate of 3.6 per million children, as compared to 0.8 per million children for aRMS

(source: ACCIS (Pastore, Peris-Bonet et al. 2006)). Furthermore, patients with eRMS fare better than aRMS patients (see Figure 1).

Both subtypes are characterised by distinct histological patterns, that were originally described in detail by Horn and Enterline based on their examinations of 39 different RMS tumours (Horn and Enterline 1958). eRMS often resemble immature skeletal muscle with long, spindle-like cells and round cells embedded in a myxoid matrix (Figure 2A), whereas the histology of aRMS is vaguely similar to foetal alveoli (Figure 2B) (Parham and Barr 2013). In the early to mid 1990s, documentation of a solid variant of aRMS led to the revision of the international classification system, encouraging the classification of aRMS based on the presence of any amount of alveolar pattern for further risk stratification (Tsokos, Webber et al. 1992; Newton, Gehan et al. 1995).

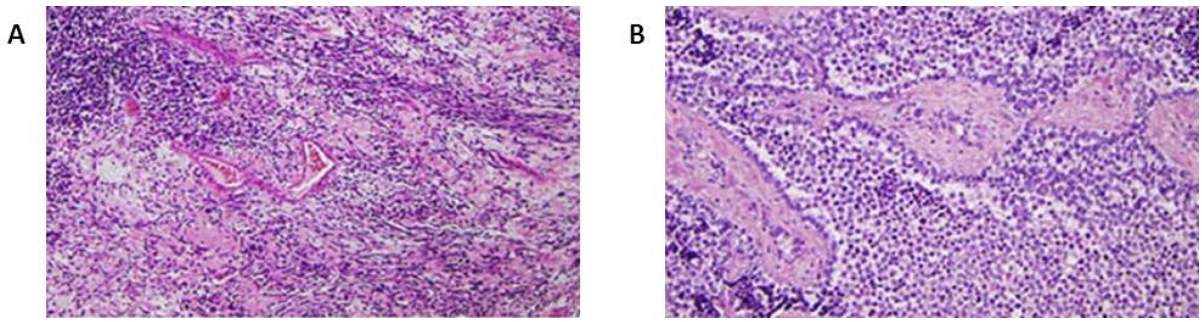

Figure 2 Histological patterns of embryonal and alveolar rhabdomyosarcoma. (A) Embryonal rhabdomyosarcoma (eRMS) mostly contain spindle cells with regions of dense and loose cellularity. (B) Alveolar RMS (aRMS) are characterised by round cells clustered into nests, thus resembling lung alveoli. *Adapted from (Parham and Barr 2013).*

Loss of heterozygosity (LOH) analysis revealed that 94% of RMS contain at least one 15 Mb region with LOH, whereby the levels of fractional allelic loss (FAL) appear to be much higher in eRMS than in other RMS subtypes (Davicioni, Anderson et al. 2009). Predominant regions of LOH were found in both arms of chromosome 11 (70% of analysed tumours) and additional affected chromosomal regions were described for 8q, 10q, 10p, 6q and 4q (Davicioni, Anderson et al. 2009). A hotspot of allelic imbalance in fusion-negative RMS is situated in the chromosome region 11p15 (Davicioni, Anderson et al. 2009), which will be discussed in the context of genetic aberrations in eRMS tumours further below.

In contrast to other tumours, mutations in the tumour suppressor gene *TP53* might be a rather rare event (Taylor, Shu et al. 2000; Ognjanovic, Martel et al. 2012), although previous studies reported that four out of six eRMS and one out of four aRMS contained *TP53* mutations (Felix, Kappel et al. 1992).

Embryonal rhabdomyosarcoma

eRMS very often appears in the head and neck and genitourinary regions, whereby the trunk and the genitourinary region represent the most favourable primary sites both with a 5-year overall survival rate of around 83% (source: ACCIS (Pastore, Peris-Bonet et al. 2006)). eRMS shows a bimodal age distribution, with highest incidence rates in children between 0-5 years of age and in adolescent males (Ognjanovic, Linabery et al. 2009).

In contrast to most aRMS, eRMS are devoid of chromosomal translocations that drive oncogenesis. Nevertheless, several common aberrant genetic features have been described in eRMS tumours. In general, eRMS tumours share a complex karyotype, characterised by severe genetic and chromosomal instability (Helman and Meltzer 2003). A recurrent attribute of eRMS tumours is the LOH in 11p15.5, which is also observed in Wilm's tumours (Scrable, Cavenee et al. 1989; Besnard-Guerin, Newsham et al. 1996). LOH in this region occurs in 72-77% of eRMS (Visser, Sijmons et al. 1997; Davicioni, Anderson et al. 2009) and was proposed to result from isodisomy of paternal origin (Scrable, Cavenee et al. 1989). This uniparental isodisomy affects several imprinted genes, resulting in potential loss of tumour suppressor *H19* and $p57^{KIP2}$ gene expression due to their natural paternal imprinting status and overexpression of the IGF2 gene, as the maternal imprinted allele is replaced by a second active paternal allele (reviewed in (Merlino and Helman 1999; Smith, Choufani et al. 2007)).

Over the recent years, evidence accumulated that eRMS tumours are hierarchically organised (Sana, Zambo et al. 2011; Walter, Satheesha et al. 2011; Ignatius, Chen et al. 2012). Furthermore, self-renewal capacity of a stem-like cancer cell subpopulation of eRMS was proposed to be controlled by hedgehog signalling (Pressey, Anderson et al. 2011), whereby expression of the downstream gene *GLI1* and the stem cell gene *NANOG* had prognostic significance (Satheesha, Manzella et al. 2015). Thus, eRMS treatment might be improved in future through therapies targeted against the cancer stem cell compartment.

Alveolar rhabdomyosarcoma

In contrast to eRMS, incidence rates of aRMS in children and adolescents appear to be equally distributed (Ognjanovic, Linabery et al. 2009) and primary sites are most often the trunk and extremities. In general, aRMS is the more aggressive subtype and most often patients present with metastasis at the time of diagnosis. The most common metastatic sites are the lungs, the bone marrow and lymph nodes (Breneman, Lyden et al. 2003).

Around 70-80% of all aRMS tumours are characterised by specific chromosomal translocations that can be detected by cytogenetic or molecular analyses (Sorensen, Lynch et al. 2002). The translocation t(2;13)(q35;q14) occurs in approximately 60-70% of aRMS, whereas in around 10% an alternative translocation t(1;13)(p36;q14) is detected (Galili, Davis

et al. 1993; Davis, D'Cruz et al. 1994). These chromosomal translocations fuse either the *PAX3* or the *PAX7* gene to the *FOXO1* gene, thereby giving rise to chimeric transcription factors named PAX3/7-FOXO1. These aberrant transcription factors contain a PAX-derived paired domain and homeodomain, the latter mediating DNA binding, at their N-terminus and a FOXO1-derived C-terminal transactivation domain (Davis, Bennicelli et al. 1995). PAX3/7-FOXO-1 positive aRMS tumours share a common gene expression profile that is distinct from that of eRMS or fusion-negative aRMS (Wachtel, Dettling et al. 2004; Davicioni, Finckenstein et al. 2006) and thus a dominant role of these fusion proteins as oncogenic driver has been suggested (Sorensen, Lynch et al. 2002; Shern, Chen et al. 2014). Furthermore, confirmation of PAX3/7-FOXO1 occurrence is an important factor for risk stratification, allowing for classification of aRMS patients into low, intermediate and high risk subgroups. Presence of PAX3-FOXO1 characterises high risk patients, as survival rates are very poor in contrast to PAX7-FOXO1 associated low risk patients, with 4-year overall survival rates of 8% and 75%, respectively (Sorensen, Lynch et al. 2002). Dismal prognosis for PAX3-FOXO1 patients correlates with a higher probability of bone marrow metastases (Sorensen, Lynch et al. 2002). Besides the very common PAX3/7-FOXO1 translocations, alternative translocations have been described in aRMS tumours; t(2;2)(q35;p23) and t(2;8)(q35;q13) give rise to PAX3-NCOA1 or PAX3-NCOA2 fusion proteins, which have similar transactivation properties as PAX3-FOXO1 (Wachtel, Dettling et al. 2004; Sumegi, Streblow et al. 2010). Additionally, fusion-negative aRMS have been reported (Barr, Qualman et al. 2002) that appear to be clinically and molecularly indistinguishable from eRMS, suggesting that the presence of fusion proteins is more crucial than the histological phenotype for classification and risk stratification (Davicioni, Anderson et al. 2009; Williamson, Missiaglia et al. 2010).

3.2. Inherited conditions

A variety of genetic disorders are known to predispose children to sarcomas, most often associated with defects in genes responsible for prevention of DNA damage (reviewed in (Parham, Alaggio et al. 2012)). For instance, germline mutations in the tumour suppressor gene *TP53* are associated with Li-Fraumeni syndrome (Srivastava, Zou et al. 1990). Patients suffering from this condition are often affected by occurrence of several tumours, especially sarcomas like rhabdomyosarcoma, throughout their life (Khayat and Johnston 2004). Other genetic disorders that have been associated with RMS are, among others, the Beckwith-Weidemann syndrome, neurofibromatosis 1, hereditary retinoblastoma and Costello syndrome (reviewed in (Slater and Shipley 2007; Parham, Alaggio et al. 2012)).

3.3. Aberrant signalling pathways

One central signalling pathway involved in RMS tumourigenesis and progression is the insulin-like growth factor (IGF) pathway. Around ten years ago it was reported that elevated activity of this signalling pathway is associated with unfavourable prognosis (Blandford, Barr et al. 2006). The main receptor tyrosine kinase involved, the insulin-like growth factor 1 receptor (IGF1R) is a transcriptional target of PAX3/7-FOXO1 (Ayalon, Glaser et al. 2001) and was also shown to be expressed on eRMS cells (El-badry, Kohn et al. 1990). Its ligand IGF2 is abundantly expressed in most RMS tumours (Scott, Cowell et al. 1985; Gray, Tam et al. 1987), where it was shown to act as an autocrine mitogen (El-badry, Kohn et al. 1990). Overexpression of IGF2 in eRMS is mostly due to LOH in the chromosomal region 11p15.5 (see section 3.1), whereas a loss of imprinting (LOI) in the same region, leading to biallelic expression, is primarily associated with IGF2 overexpression in aRMS (Zhan, Shapiro et al. 1994). Therefore, increased IGF2 and IGF1R levels are key to a strong mitogenic signalling loop that promotes sustained proliferation as well as survival of RMS cells.

Several other receptor tyrosine kinase receptors besides IGF1R play a role in RMS progression (reviewed in (Crose and Linardic 2011) and, like IGF1R, some of them are also direct transcriptional targets of the PAX3/7-FOXO1 fusion protein, including MET, the platelet derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor 1 (VEGFR1) and fibroblast growth factor receptor 4 (FGFR4) (Epstein, Shapiro et al. 1996; Epstein, Song et al. 1998; Barber, Barber et al. 2002; Cao, Yu et al. 2010). Their involvement in RMS progression has been confirmed by numerous studies (reviewed in (Crose and Linardic 2011). For instance, MET in combination with its ligand, the hepatocyte growth factor (HGF), not only regulates migration and metastasis of RMS, but MET/HGF signalling might also play a role in promoting proliferation, as a MET knockdown led to decreased cell proliferation and tumour burden (Taulli, Scuoppo et al. 2006; Lukasiewicz, Miekus et al. 2009).

One of the more recently identified receptor tyrosine kinases of importance in RMS tumour growth and metastasis is FGFR4. Elevated mRNA levels of FGFR4 have been found in all aRMS cases (Wachtel, Dettling et al. 2004; Davicioni, Finckenstein et al. 2006) where it could be shown that FGFR4 is a direct target of PAX3-FOXO1 (Cao, Yu et al. 2010). Further investigations reported activating mutations in FGFR4 in up to 20% of RMS (Taylor, Cheuk et al. 2009; Paulson, Chandler et al. 2011) and amplifications of the *FGFR4* gene in around 20% of eRMS (Paulson, Chandler et al. 2011). The study of Taylor et al. further demonstrated the oncogenic role of mutationally active FGFR4 in RMS cell lines (Taylor, Cheuk et al. 2009). Wachtel et al. identified subgroups of aRMS cells in which stimulation of FGFR4 signalling, through application of basic fibroblast growth factor (bFGF), rescues from IGF1R-PI3K-mTOR inhibition induced apoptosis (Wachtel, Rakic et al. 2014). These cells mediated proapototic

stimuli via the BH3-only proteins Bim and Bad, whereas apoptosis induction in non-rescue cells was Bmf-dependent. These data suggest that a subset of FGFR4-sensitive aRMS, characterised through low levels of the PAX3-FOXO1 target gene AP2β, will very likely benefit from FGFR4 inhibition for improvement of current RMS treatment modalities (Wachtel, Rakic et al. 2014).

3.4. Treatment of rhabdomyosarcoma

During the last 50 years, therapy plans for treatment of RMS have evolved significantly. Nowadays all patients receive intensive, multi-agent chemotherapy, which is most often accompanied by radiotherapy and in some cases by surgical resection of the tumour. One of the most common chemotherapeutic regimen is referred to as VAC and combines the three drugs vincristine, actinomycin-d and cyclophosphamide. Other drugs that are sometimes included are irinotecan or topotecan, ifosfamide, etoposide and doxorubicin. The general VAC regimen dates back to the early seventies, however constant efforts are being made to improve treatment plans, e.g. by testing different drug combinations or optimising dosages of employed drugs (review of recent trials (Hawkins, Gupta et al. 2014)). Furthermore, better risk stratification helps to tailor treatment strategies to individual patients (Parham and Barr 2013; Hawkins, Gupta et al. 2014). However, survival rates stagnated or barely improved over the last years and are still particularly low for metastatic RMS (Ognjanovic, Linabery et al. 2009; Van Gaal, Van Der Graaf et al. 2012). Additionally, chemo- and radiotherapy might lead to long term side effects in children, including deafness and secondary malignancies (Robison and Hudson 2014). Therefore, novel therapies, e.g. involving recently identified targets (see section 3.3), are required to complement current treatment options.

In order to improve existing therapy modalities, many targeted agents currently undergo clinical testing in paediatric sarcomas (reviewed in (Anderson, Denny et al. 2012). As direct targeting of transcription factors like PAX3/7-FOXO1 remains a very challenging task, the focus of targeted therapies for treatment of RMS has been put on specific signalling pathways that promote RMS progression *in vitro* and *in vivo* (see section 3.3). For instance, targeting the IGF pathway represents a promising approach to treat RMS tumours. Different ways to disrupt IGF1R signalling in RMS cells have been explored, including IGF1R anti-sense RNA (Shapiro, Jones et al. 1994), IGF1R blocking antibodies (Kalebic, Tsokos et al. 1994) and the application of the selective inhibitor NVP-AEW451 (Scotlandi, Manara et al. 2005). Blocking IGF1R signalling causes cell cycle arrest in G1 and apoptosis in highly sensitive cells (Scotlandi, Manara et al. 2005), whereby sensitivity to inhibition correlates with levels of IGF1R in RMS cells (Cao, Yu et al. 2008). Thus, Cao et al. showed that Rh4 and

CTR were the most sensitive RMS cell lines, while RD, Rh30 and RMS13 were moderately sensitive to treatment with an anti-IGF1R antibody (Cao, Yu et al. 2008). Combined use of IGF1R and mTOR inhibitors proved to suppress a rapamycin treatment-induced Akt feedback loop and was therefore proposed to be of benefit for the treatment of RMS and other IGF-driven tumours (Wan, Harkavy et al. 2007; Cao, Yu et al. 2008; Kurmasheva, Dudkin et al. 2009). Currently, several phase I and II clinical trials investigating the use of monoclonal IGF1R antibodies for therapy of RMS are being conducted, with a focus on the compound cixutumumab (clinicaltrials.gov, search term “IGF1R AND rhabdomyosarcoma”) (McKian and Haluska 2009).

4. Proprotein convertases

Most proteins are modified during or after protein biosynthesis. These post-translational modifications increase the diversity of proteins encoded by the genome of an organism. Over 200 posttranslational modifications are currently known, including serine, threonine or tyrosine phosphorylation; cholesterol attachment; cysteine palmitoylation; lysine methylation and ubiquitination; protein oxidation or sumoylation; and asparagine and serine/threonine glycosylation. Many of these processes are carried out by specific enzymes. A frequent irreversible posttranslational modification is the limited proteolysis of precursor proteins. It has been estimated that the human degradome comprises over 550 distinct proteases that can be classified into five major classes: aspartic, metallo-, cysteine, serine and threonine proteases (reviewed in (Puente, Sanchez et al. 2003)). Among these enzymes serine proteases seem to be the most abundant as over 200 serine proteases in humans have been identified, representing ~1% of all proteins (Puente, Sanchez et al. 2003; Long and Cravatt 2011). Serine proteases can be divided into two main families depending on their relation to either trypsin/chymotrypsin or bacterial subtilisin. The latter are often referred to as subtilases and can be further subdivided into the prokaryotic subfamily S8A and the eukaryotic subfamily S8B, comprising the archetype yeast kexin and the proprotein convertases (PCs) (Siezen and Leunissen 1997; Rawlings, Barrett et al. 2012). The family of PCs can be further subdivided into the seven basic amino acid-specific members: PC1/3, PC2, furin, PC4, PC5, PACE4 (paired amino acid cleaving enzyme 4) and PC7; and two members, SKI-1 (subtilisin kexin isozyme 1; also known as S1P) and PCSK9 (proprotein convertase subtilisin kexin 9), that have no preference for basic amino acid motifs for cleavage.

4.1. Discovery

The first cornerstone for the identification of PCs was laid in 1984 with the discovery of the yeast convertase kexin that cleaves the precursor of pro-K1 killer toxin and pro-α-factor of the yeast *Saccharomyces cerevisiae* C-terminally of ArgArg↓ and LysArg↓ basic residue pairs (Julius, Brake et al. 1984; Fuller, Sterne et al. 1988). Shortly thereafter, the product of the *fur* gene, furin, was proposed to be a mammalian homologue of kexin, mainly based on their striking similarity (van de Ven, Voorberg et al. 1990). Simultaneously, two other PCs, PC1/3 and PC2, were discovered (Seidah, Gaspar et al. 1990; Smeekens and Steiner 1990; Smeekens, Avruch et al. 1991). Within the following five years four more PCs were discovered: PC4 (Nakayama, Hosaka et al. 1991; Seidah, Day et al. 1992), PC5 (Lusson, Vieau et al. 1993; Nakagawa, Murakami et al. 1993), PACE4 (Kiefer, Tucker et al. 1991) and PC7 (Meerabux, Yaspo et al. 1996; Seidah, Hamelin et al. 1996). These seven PCs with a

cleavage preference for basic amino acid residues are known as furin-like PCs and represent the core members of the PC family. Two more subtilisin-related proteases have been described: the pyrolysin-like SKI-1 (Espenshade, Cheng et al. 1999; Seidah, Mowla et al. 1999); and the proteinase K-like PCSK9 (Seidah, Benjannet et al. 2003). Thus, the family of PCs contains nine subtilisin-like secretory serine proteases and their respective genes are called *PCSK1-PCSK7*, whereby the gene encoding furin (PCSK3) is officially named *FURIN*.

4.2. Structure & biochemistry

Structural features

All PCs share a high structural homology. Their N-terminal part is the most conserved region and consists of common domains that serve discrete biochemical functions, whereas the C-terminal displays more variability among PCs (Figure 3).

The N-terminal part encompasses three to four important domains: the signal peptide, the prodomain, the catalytic domain and, in the case of the furin-like PCs, the P domain. The signal peptide at the very N-terminus directs translocation into the endoplasmatic reticulum (ER) and is subsequently cleaved and released by signal peptidases. The signal peptide is followed by a prosegment that essentially acts as a molecular chaperone and inhibitor of PCs. Its role in the zymogen activation mechanism will be discussed in detail below. The most conserved domain is the catalytic domain, sharing 54-70% sequence identity across all PCs as compared to furin (Siezen and Leunissen 1997; Thomas 2002). A catalytic triad consisting of the amino acids Asp, His and Ser is required for enzymatic activity, as is the presence of an Asn residue, or Asp in the case of PC2, providing the oxyanion hole during the hydrolyses reaction. The active site will be discussed in detail further below. The P domain is located C-terminally from the catalytic domain in all basic amino acid-specific PCs and plays an important role in ensuring proper folding of the proteins and in maintaining stability and activity. Another function of the P domain is the regulation of pH and calcium dependence (Zhou, Martin et al. 1998). Furthermore, all PCs but PC7 contain an RGD motif within the P domain. Despite first investigations revealing that mutations in the RGD motif of PC1/3 lead to a loss of function and intracellular relocation (Lusson, Benjannet et al. 1997), the exact function of this conserved motif is still poorly understood.

In contrast to the well conserved N-terminus, the C-terminal part is more specific to individual PCs and regulates their intracellular localisation and trafficking. The C-terminal part of furin, PC5 and PACE4 for instance includes a Cys-rich domain (CRD). For PC5 and PACE4 it has been shown that this region allows cell surface anchoring via binding of heparan sulphate proteoglycans (HSPGs) or tissue inhibitors of metalloproteases (TIMPs), thus favouring the proteolytic processing of their cognate surface-tethered substrates (Nour, Mort et

al. 2005; Mayer, Hamelin et al. 2008). The three transmembrane PCs furin, PC5B and PC7 present a unique transmembrane domain that directs localisation to the membrane and sorting to cellular compartments. PCSK9 harbours a unique Cys-His-rich domain (CHRD) that is necessary to target the extracellular low density lipoprotein receptor (LDLR) for degradation (Saavedra, Day et al. 2012).

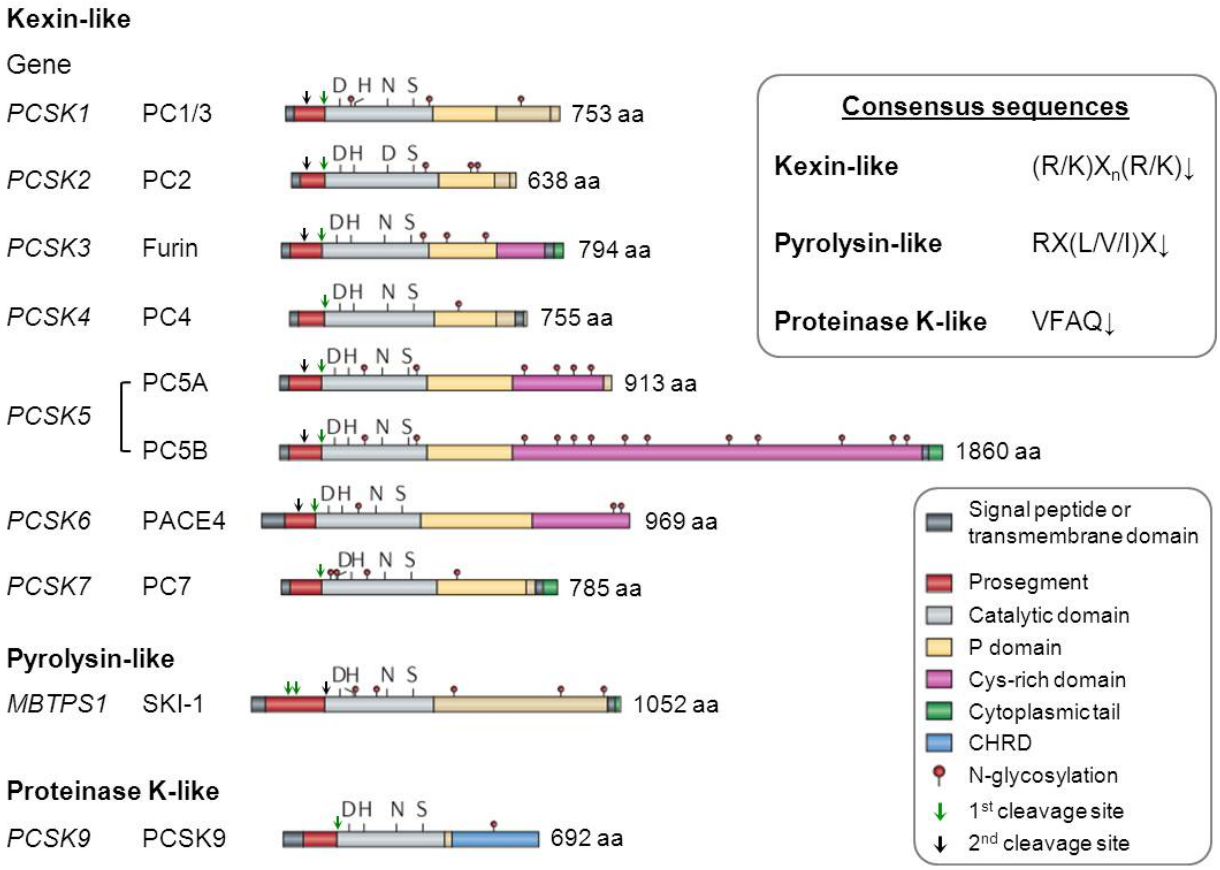

Figure 3 Schematic representation of the primary structures of human proprotein convertases.
The different domains, positions of N-glycosylations and primary (green arrows), as well as secondary autocatalytic cleavage sites (black arrows) are depicted. PC4, PC7 and PCSK9 contain only a primary site. All PCs contain a signal peptide, a prosegment and a catalytic domain, whereby presence of a β-barrel P domain is limited to the kexin-like PCs. The catalytic triad residues Asp, His and Ser and the oxyanion hole residue Asn (Asp for PC2) are indicated within the catalytic domain. The distinct C-terminal domains contain unique sequences regulating localisation and trafficking of the individual PCs. PC5A and PC5B represent the two validated alternative splice variants of PC5. *Modified from (Seidah and Prat 2012)*

Activation of proprotein convertases

All PCs undergo a well-orchestrated zymogen activation mechanism consisting of up to two autocatalytic cleavage steps and other posttranslational modifications (Figure 4). This multi-step mechanism has been first described for the activation of furin (Anderson, VanSlyke

et al. 1997). All PCs but PC2 are co-translationally modified in the ER, where a signal peptidase cleaves and releases the signal peptide and N-glycosylations are introduced at various sites. The N-terminal prosegment acts as a chaperone and inhibitor and guides proper folding of the proprotein convertases into an active conformation within the ER lumen. Hence, the primary cleavage site becomes accessible, allowing intramolecular autocatalytic cleavage of the prosegment. The prosegment remains attached to the mature protease, keeping it in an inactive state. Initial cleavage of the prosegment is required for release of the complex from the ER (Creemers, Vey et al. 1995). Different than for the other PCs, primary cleavage within the prosegment of SKI-1 occurs at two internal sites, called B and B' (Espenshade, Cheng et al. 1999; Elagoz, Benjannet et al. 2002). With the exception of PC4 and PC7 a secondary autocatalytic cleavage step within the prosegment is required to release the inhibitory segment (Anderson, Molloy et al. 2002). PCs requiring this secondary cleavage step display distinct pH and calcium optima for this last cleavage step, ensuring that activation takes place in selected intracellular compartments (Seidah, Mayer et al. 2008). Thus, PC1/3 and PC2 are generally activated in immature secretory granules of the regulated secretory pathway. Furin is primarily activated in the *trans*-Golgi network (TGN), whereby PACE4 and PC5 may be activated both in the TGN and on the cell surface. SKI-1 is fully activated in the *cis*- and *medial*-Golgi.

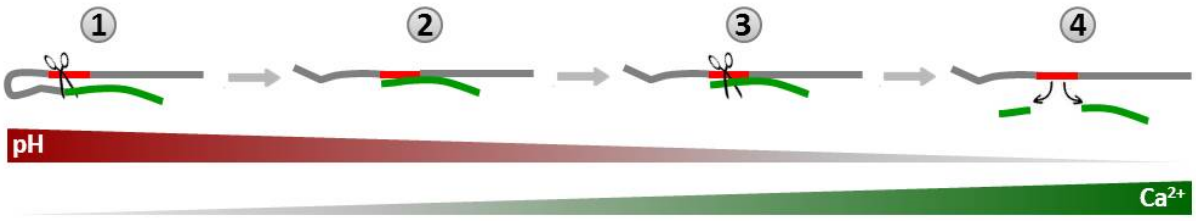

Figure 4 Schematic representation of the autocatalytic activation mechanism of proprotein convertases.
Proprotein convertases (PCs) are translated as inactive zymogens that undergo a multi-step activation process involving one or two autocatalytic cleavage steps. In the endoplasmatic reticulum (ER), pro-PCs are subject to folding events and removal of the signal peptide. Subsequently, the catalytic domain (red) cleaves the prosegment (green) (1). The prosegment remains attached, keeping the PC in an inactive state (2). Upon shuttling to the TGN a secondary autocatalytic cleavage within the prosegment takes place (3), leading to the release of this inhibitory chaperone molecule and subsequent activation of the mature PC (4). PC4, PC7 and PCSK9 do not require this second step for activation.

Similar to PC4 and PC7, PCSK9 undergoes only one autocatalytic cleavage step, however, other than in all other PCs, the protein remains bound to the prosegment (Seidah, Benjannet et al. 2003; Benjannet, Rhainds et al. 2004). Association to its prosegment keeps PCSK9 in an enzymatically inactive state (Cunningham, Danley et al. 2007). As PCSK9's

chaperone function on LDLR is independent of its catalytic activity, PCSK9 itself represents its only known enzymatic target (McNutt, Lagace et al. 2007).

PC2 does not follow the aforementioned autocatalytic cleavage mechanism. proPC2 is bound to its ~30 kDa binding protein 7B2 and the complex is transported to acidic immature secretory granules. The protein 7B2 was first described in 1982 (Hsi, Seidah et al. 1982) and has been reported to act as a chaperone for proPC2 maturation and activity (Braks and Martens 1994). Thus, it facilitates intracellular transport of PC2 and additionally inhibits PC2 whilst being bound. Autocatalytic cleavage of the prodomain of proPC2 and subsequent dissociation of the prodomain and 7B2 occur in the acidic environment of the secretory granules.

The consensus cleavage sequence of proprotein convertases

Due to their similarity to furin, the seven basic amino acid-specific PCs are often referred to as “furin-like PCs”. These seven core members exclusively process motifs that require the presence of basic flanking arginine (R) or lysine (K) residues and thus the most common consensus cleavage site is R/K-X_n-R/K↓, whereby X represents any amino acid but cysteine and rarely proline, n represents the numbers 0, 2, 4 or 6, and the arrow stands for the cleavage site. The sequence of the recognition site is based on analysis of various substrates and was first described in the early 1990’s, among others for furin-mediated cleavage of anthrax protective antigen (Molloy, Bresnahan et al. 1992). Despite the similarities in sequence preference, certain differences exist. For instance, furin seems to favour the presence of arginine at position P4 and arginine or lysine at position P2, thus preferentially recognizes a site represented by R-X-R/K-R (Hosaka, Nagahama et al. 1991). The preferences of furin for arginine in P1 as well as for basic amino acids at P2 and P4 are explained by the structure of its catalytic domain, which was solved in 2003 by Henrich et al. (Henrich, Cameron et al. 2003) and will be discussed in detail in section 5.1. Since less favourable residues at P1 and P4 may be compensated for by favourable residues at P2 and P6 and some amino acids are better tolerated than others at P1’ and P2’, the use of a neural network instead of a strict consensus sequence for prediction of PC substrates has been proposed (Duckert, Brunak et al. 2004). Such furin- or PC-specific networks trained with extensive datasets of known substrates would allow identifying substrates that do not harbour the stringent consensus cleavage site.

The remaining two PCs, SKI-1 and PCSK9, do not cleave after basic amino acid residues. SKI-1 cleaves its substrates after non-basic residues carboxyterminally of the recognition sequence R-X-(L/V/I)-X↓ (Seidah, Mowla et al. 1999; Toure, Munzer et al. 2000).

PCSK9 on the other hand processes itself at its internal sequence VFAQ↓ (Naureckiene, Ma et al. 2003).

4.3. Subcellular localisation and tissue distribution

All PCs display distinct tissue expression and subcellular localisation (Table 1), a feature that determines their selectivity towards distinct substrates.

Furin, PC7 and PC5B can cleave their substrates within the constitutive secretory pathway. They are widely expressed and can cycle from the TGN to the cell surface and back through endosomes (Molloy, Anderson et al. 1999; Xiang, Molloy et al. 2000; Rousselet, Benjannet et al. 2011). Nevertheless, these three PCs display distinct intramolecular sorting motifs and/or additional trafficking routes. For instance, while the majority of mature PC7 cycles to the cell surface within the constitutive secretory pathway, a fraction of PC7 reaches the cell surface through an unconventional secretory pathway that is brefeldin A- and COPII-independent (Rousselet, Benjannet et al. 2011). Recycling of PC7 from the cell surface via clathrin-coated vesicles seems to be dependent on the cytoplasmic region between Ala^{713} and Cys^{726} (Declercq, Meulemans et al. 2012). Trafficking of furin will be discussed in detail in the section 5.1. In contrast to the membrane-bound PC5B, the alternative splice variant PC5A is secreted into the extracellular milieu (Lusson, Vieau et al. 1993).

PC1/3 and PC2 are exclusively expressed in neuroendocrine cells. Processing of PC1/3 and PC2 prohormone substrates takes place within immature and dense-core secretory granules as part of the acidic regulated secretory pathway. Due to their requirement for a low pH, optimal pH range for PC1/3 and PC2 lies around 5.5-6.5 and 5.5-6.0, respectively, processing of substrates outside the cell is highly unlikely (Jean, Basak et al. 1993; Shennan, Taylor et al. 1995).

The tissue expression of PACE4 is widespread and abundant amounts of this enzyme can be found in the anterior pituitary, the heart, the liver, the kidney, the digestive system, the placenta and the brain (Seidah, Chretien et al. 1994; Tsuji, Sakurai et al. 2003). Like PC5A, PACE4 can be transported from the TGN to the cell surface and be retained at the cell surface and/or extracellular matrix (ECM) through binding to HSPGs or TIMPs (Tsuji, Sakurai et al. 2003; Mayer, Hamelin et al. 2008).

PC4 seems to be exclusively expressed in germ cells. Thus, its expression was confirmed in testicular germ cells in males (Seidah, Day et al. 1992; Gyamera-Acheampong, Tantibhedhyangkul et al. 2006) and in the placenta and ovary in females (Gyamera-Acheampong and Mbikay 2009). Its exact intracellular localisation remains elusive.

The pyrolysin-like PC SKI-1 is ubiquitously expressed (Seidah, Mowla et al. 1999). It is mostly localised in the *cis*- and *medial*-Golgi and generally does not reach the cell surface. Furthermore, SKI-1 can be sorted to endosomal/lysosomal compartments for degradation (Pullikotil, Benjannet et al. 2007).

Expression of PCSK9 is mostly found in adult liver, small intestine and kidney (Seidah, Benjannet et al. 2003; Zaid, Roubtsova et al. 2008). PCSK9 is secreted into the extracellular milieu, whereby hepatocytes in the liver represent the main source of circulating PCSK9 (Zaid, Roubtsova et al. 2008). Upon binding to LDLR at the cell surface, the complex is internalised into endosomes and forwarded to lysosomes for degradation (Maxwell, Fisher et al. 2005; Nassoury, Blasiole et al. 2007).

Table 1 Expression and subcellular localisation of proprotein convertases.

Proprotein convertase	Number of amino acids	Tissue distribution	Subcellular localisation	Secreted
PC1/3	753	Neuroendocrine	Acidic regulated secretory granules	Secreted
PC2	638			
Furin	794	Ubiquitous	TGN, cell surface, endosomes	Shed
PC4	755	Germinal	Cell surface?	Shed
PC5	913 (PC5A) 1860 (PC5B)	Widespread: adrenal cortex, intestine, ovary, kidney	Cell surface, ECM	Secreted PC5A; shed PC5B
PACE4	969	Widespread: muscle, heart, pituitary, kidney, intestine, cerebellum	Cell surface, ECM	Secreted
PC7	785	Ubiquitous	TGN, cell surface, endosomes	Not secreted
SKI-1	1052	Ubiquitous	*cis*- and *medial*-Golgi	Not secreted
PCSK9	692	Liver, intestine, kidney	TGN, extracellular	Secreted

ECM, extracellular matrix; PACE4, paired basic amino acid cleaving enzyme 4; PC1/3, proprotein convertase 1/3; PCSK9, proprotein convertase subtilisin kexin 9; SKI-1, subtilisin kexin isozyme 1; TGN, *trans*-Golgi network. *Modified from (Seidah and Prat 2012).*

4.4. Proprotein convertases in health and disease

Proprotein convertases cleave a huge variety of proproteins. Cleavage redundancy towards substrates might occur because of overlapping cleavage motif preferences, tissue expression and subcellular localisation. Redundancy in substrate processing was observed in *in vitro* and *in vivo* studies, especially between furin, PC5 and PACE4 and to a lesser extent

for PC7 (reviewed in (Seidah, Mayer et al. 2008)). Nevertheless, time and spatial resolution, i.e. localisation of a PC and a substrate at a given time, are key determinants that have to be assessed *in vivo*. A collection of the various PC substrates can be found in the MEROPS Peptidase Database (https://merops.sanger.ac.uk/ (Rawlings, Barrett et al. 2012)). A choice of key substrates is presented in Table 2.

Table 2 Substrates of proprotein convertases.

PC	Typical substrates	Comments
Kexin-like	**$(R/K)X_n(R/K)\downarrow$**	
PC1/3	PC-1 specific: GHRH, ACTH, GLP1, GLP2	Often collaborates with PC2 (e.g. for insulin, TRH and MCH production)
PC2	PC2-specific: β-endorphin, glucagon and α-MSH	Often collaborates with PC1/3
Furin	• Growth factors (TGFβ, IGF1, IGF2) • Receptors (insulin receptor, IGF1R) • Adhesion molecules (α5 integrin, RGMA) • Metalloproteases (MT1-MMP) • Viral glycoproteins (HIV gp160) • Bacterial toxins (anthrax pa83)	Strong *in vitro* and *ex vivo* redundancy with PC5 and PACE4
PC4	IGF2 and PACAP	Cleaves substrates in germ cells
PC5	• Growth factors (GDF11) • Receptors (PTPRM) • Adhesion molecules (α5 integrin, neuronal L1CAM)	Strong *in vitro* and *ex vivo* redundancy with furin and PACE4
PACE4	• Growth factors (Nodal and Lefty) • Metalloproteases (ADAM-TS4) • Viral glycoproteins (HIV protein Vpr)	Strong *in vitro* and *ex vivo* redundancy with furin and PC5
PC7	Receptors (transferrin receptor 1)	Partial redundancy with furin, PC5 and PACE
Pyrolysin-like	**$RX(L/I/V)X\downarrow$**	
SKI-1	• Transcription factors (SREBPs, ATF6, CREBs) • GlcNAc-1-phosphotransferase, RGMA • Viral glycoproteins (Lassa virus glycoprotein, CCHF Gn)	Cleaves substrates in *cis*- and *medial*-Golgi
Proteinase K-like	**$VFAQ\downarrow$**	
PCSK9	PCSK9 only	Interacting proteins: LDLR, VLDLR, LRP8

α-MSH, α-melanocyte-stimulating hormone; ACTH, adrenocorticotropic hormone; ADAM-TS4, a disintegrin and metalloprotease with thrombospondin motif 4; ATF6, activating transcription factor 6; CCHF Gn, Crimean–Congo haemorrhagic fever virus glycoprotein Gn; CREB, cyclic AMP-responsive element binding protein; GDF11, growth differentiation factor 11; GHRH, growth hormone-releasing hormone; GlcNAc, N-acetylglucosamine; GLP1, glucagon-like peptide 1; gp160, envelope glycoprotein 160; IGF1/2, insulin-like growth factor 1/2; IGF1R, insulin-like growth factor 1 receptor; L1CAM, L1 cell adhesion molecule; LDLR, low-density lipoprotein receptor; LRP8, LDLR-related protein 8; MCH, melanin concentrating hormone; MT1-MMP, membrane-type 1 matrix metalloprotease; pa83, protective antigen 83; PACAP, pituitary adenylyl cyclase-activating peptide; PACE4, paired basic amino acid cleaving enzyme 4; PC1/3,

proprotein convertase 1/3; PCSK9, proprotein convertase subtilisin kexin 9; PTPRM, protein tyrosine phosphatase receptor type M; RGMA, repulsive guidance molecule A; SKI-1, subtilisin kexin isozyme 1; SREBP, sterol regulatory element-binding protein; TGFβ, transforming growth factor-β; TRH, thyrotropin releasing hormone; VLDLR, very-low-density lipoprotein receptor. *Modified from.(Seidah and Prat 2012).*

Based on their involvement in the activation and expression of a vast number of substrates, the function of PCs has been implied in the development and progression of many pathological disorders and infectious diseases (reviewed in (Artenstein and Opal 2011)). The role of PCs in cancer will be discussed in section 4.5.

Deciphering the roles of PCs: use of knockout mice

In order to decipher the role of distinct PCs in tissues and organs, knockout mice have been generated resulting in *in vivo* defects that have been studied in detail (reviewed in (Scamuffa, Calvo et al. 2006)). From these studies it emerged that PCs have an important function in embryogenesis and organ development. Decreased expression or lack of expression of PCs can affect several programmes of organogenesis. Particularly furin, PC5 and SKI-1 null mice show an increased rate of embryonic mortality (Roebroek, Umans et al. 1998; Yang, Goldstein et al. 2001; Essalmani, Hamelin et al. 2006). Knockout of PC1/3 or PC2 results in metabolic or endocrine disorders and developmental defects or growth retardation (Furuta, Yano et al. 1997; Zhu, Zhou et al. 2002). PACE4 null mice have a high viability rate of 75%, but are often born with craniofacial abnormalities (Constam and Robertson 2000). The knockout of PC4 decreases fertility of the mice (Mbikay, Tadros et al. 1997), while absence of PC7 does not have any apparent consequences. The insights gained from partial or complete knockouts of furin will be discussed in more depth in section 5.1.

Hormones and endocrinopathies

Mice that are deficient in PC1/3 or PC2 are viable but display complex endocrinologic disturbances. They show irregular processing of hormones in the pro-opiomelanocortin (POMC) pathway, abnormalities concerning neuroendocrine function and impaired maturation of pro-glucagon and pro-insulin, leading to glucose homeostasis defects (Furuta, Yano et al. 1997; Zhu, Zhou et al. 2002). Several congenital cases of PC1 deficiency have been described in humans. They recapitulate some of the phenotypical features observed in PC1/3 knockout mice, such as abnormal glucose homeostasis or impaired prohormone processing, but in contrast to mice, the affected subjects had a normal growth and suffered from early-onset obesity (O'Rahilly, Gray et al. 1995; Jackson, Creemers et al. 1997; Jackson, Creemers et al. 2003; Farooqi, Volders et al. 2007). In the first case ever described, the female patient harboured two mutations in both PC1/3 alleles: a heterozygous missense mutation of Gly593Arg, leading to improper processing of PC1/3 and retention in the ER, and a

heterozygous A→C transversion at the +4 donor splice site of intron 5, causing exon skipping, a frameshift and generation of a premature stop codon (Jackson, Creemers et al. 1997). In a different case, a homozygous missense mutation Ser307Leu was described resulting in impaired autocatalytic activity of PC1/3 (Farooqi, Volders et al. 2007). Furthermore, several studies have shown that mutations in the *PCSK1* gene can be the cause of monogenic obesity (Benzinou, Creemers et al. 2008; Corpeleijn, Petersen et al. 2010). Being a carrier of a heterozygous mutation in *PCSK1* that (partially) disables the function of the PC1/3 enzyme increases the risk of obesity by 8.7 fold compared to wild type carriers (Creemers, Choquet et al. 2012).

Alzheimer's disease

The WHO estimates that 60-70% of the 47.5 million dementia cases worldwide can be attributed to Alzheimer's (WHO 2015). Alzheimer's disease is a neurodegenerative disease leading to deterioration of cognitive functions. The first symptoms often manifest in people over age 65, nevertheless, 4-5% of the patients present with early-onset Alzheimer's disease (reviewed in (Mendez 2012)). It has been shown that furin and/or furin-like PCs are the proteases responsible for the conversion of pro-BACE (β-site amyloid precursor protein cleaving enzyme) and have thus been directly linked with amyloid plaque formation in Alzheimer's disease (Bennett, Denis et al. 2000). Amyloid plaques, as well as neurofibrillary tangles represent the two pathological core hallmarks of Alzheimer's disease (reviewed in (Ballard, Gauthier et al. 2011)). Neurofibrillary tangles are formed by aggregates of the microtubule-associated protein tau, leading to disruption of microtubules inside nerve cell bodies. Pathological or toxic tau is thought to arise from hyperphosphorylation of normal tau, however underlying mechanism are still poorly understood (reviewed in (Iqbal, Alonso Adel et al. 2005)). Around 25 years ago another hypothesis emerged postulating that the deposition of extracellular amyloid beta (Aβ) might be the main cause of Alzheimer's disease (Hardy and Allsop 1991). Cleavage of amyloid precursor protein (APP) into the Aβ peptides $A\beta_{40}$ and $A\beta_{42}$ is catalysed by the secretase enzymes BACE and γ-secretase (reviewed in (Vassar, Kovacs et al. 2009)). An increased ratio in favour of $A\beta_{42}$ is thought to trigger formation of amyloid plaques, as $A\beta_{42}$ aggregates more readily (Jarrett, Berger et al. 1993). As mentioned above, the APP cleaving enzyme BACE in turn requires PC mediated proteolytic removal of its prodomain to form the mature enzyme. Interestingly, alternative cleavage of APP resulting in a secreted APPα fragment was suggested to be catalysed either by furin or by PC7 (Lopez-Perez, Seidah et al. 1999; Hwang, Kim et al. 2006), pointing at opposing roles of PCs in amyloid plaque formation as increased APPα fractions are considered to lower potentially

harmful Aβ fractions. Nevertheless, the exact contribution of PC activity to the onset of Alzheimer's disease still remains elusive.

Infectious diseases

The action of PCs has been implied in the activation of many bacterial and viral pathogens. For instance, three distinct forms of bacterial toxins are known to require processing of furin or furin-like PCs for their activation. Single-chain toxins, which are composed of a toxic subunit and a binding subunit, are cleaved within endosomes. Prominent examples are the furin-catalysed conversion of pseudomonas exotoxin A (Gu, Gordon et al. 1996), diphtheria toxin (Tsuneoka, Nakayama et al. 1993) and Shiga toxins (Lea, Lord et al. 1999). The second group of bacterial toxins that are cleaved by PCs is called binary toxins and is represented by the anthrax toxin protective antigen (PA) (reviewed in (Young and Collier 2007)). Upon binding of PA_{83} to receptors on the cell surface, an N-terminal 20 kDa fragment, PA_{20}, is removed proteolytically by furin to generate the active PA_{63} moiety (Klimpel, Molloy et al. 1992; Molloy, Bresnahan et al. 1992). PA_{63} is then able to self-associate to form the prepore and to form complexes with up to three moieties of lethal factor (LF) and/or edema factor (EF), leading to endocytosis of the complex. Ultimately, this results in the release of LF and EF into the cytosol where these two enzymes act on cytosolic substrates. Even though furin seems to be the main activator of PA_{83}, PACE4 was proposed as an alternative cleaving enzyme (Gordon, Rehemtulla et al. 1997). In the case of the soluble bacterial toxin aerolysin, secreted by *Aeromonas hydrophila*, it could be shown that proteolytic cleavage of soluble proaerolysin through furin, PACE4 or PC5A on the cell surface is required for subsequent pore formation and entry into host cells (Abrami, Fivaz et al. 1998).

Many envelop glycoproteins of viral pathogens also require proteolytic cleavage before their mature forms can fuse with the host cell membrane. Many viruses utilise furin or other host PCs for this purpose. For instance, the conversion of the human immunodeficiency virus (HIV) 1 glycoprotein 160 (gp160) into gp120 and the fusiogenic gp41 is promoted by PCs (reviewed in (Moulard and Decroly 2000)). Furin was thought to be the central endoprotease in this process (Hallenberger, Bosch et al. 1992), however gp160 can be efficiently cleaved in the furin-deficient LoVo cell line (Ohnishi, Shioda et al. 1994), suggesting the presence of alternative gp160 cleaving enzymes. In the case of influenza A viruses the haemagglutinin glycoprotein undergoes proteolytic activation. It has been shown that the virulence of influenza virus directly correlates with the presence of a PC cleavage motif (Decha, Rungrotmongkol et al. 2008). Other examples of (potentially) deadly viruses that rely on PC activity are measles (Watanabe, Hirano et al. 1995) and the two highly pathogenic filoviruses Marburg (Volchkov, Volchkova et al. 2000) and Ebola (Volchkov, Feldmann et al. 1998). Similar to influenza viruses the absence of a PC cleavage site, as in the Ebola Reston subtype, conveys reduced pathogenicity in humans (Volchkov, Feldmann et al. 1998).

Lipid disorders and atherosclerosis

PCSK9 has a unique role in protein degradation of the hepatic low density lipoprotein receptor (LDLR). Independent of its intrinsic enzymatic activity, it binds tightly to cell surface LDLR, thereby mediating internalisation and subsequent lysosomal degradation (McNutt, Lagace et al. 2007; Zhang, Garuti et al. 2008). As low density lipoprotein (LDL) cholesterol is primarily cleared by binding to LDLR and subsequent receptor-mediated endocytosis, removal of cell surface LDLR leads to elevated levels of circulating LDL cholesterol (Maxwell and Breslow 2004). Accordingly, mutations in PCSK9 that increase its affinity for LDLR have been associated with autosomal dominant hypercholesterolaemia (ADH) (Abifadel, Varret et al. 2003; Allard, Amsellem et al. 2005). The approaches and the potential of inhibiting the function of PCSK9 in order to treat hypercholesterolaemia will be discussed in section 4.6. In contrast to PCSK9's function in hypercholesterolaemia, frequent loss-of-function mutations leading to hypocholesterolaemia have equally been described. The two nonsense mutations Y142X and C679X, found in 2% of African Americans, were associated with 40% lower LDL cholesterol plasma levels and it was hypothesised that carriers of such mutations exhibit substantially lower risk for coronary heart disease (Cohen, Pertsemlidis et al. 2005; Kotowski, Pertsemlidis et al. 2006).

4.5. Proprotein convertases and cancer

Over the last 20 years evidence accumulated linking the activity of PCs to the genesis and progression of multiple proliferative disorders, including cancer. Initial hypotheses were based on their role in processing and activation of proteins directly involved in the formation of neoplasias. Later, these were expanded by direct investigations of PC expression and loss- or gain-of-function studies in different human cancer models and tumour cell lines (reviewed in (Khatib, Siegfried et al. 2002; Bassi, Fu et al. 2005)). Their expression inside a tumour can be assessed either at the protein level by immunohistochemical staining and immunoblotting, or at the mRNA level by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), Northern blot and *in situ* hybridisation. However, examination of enzymatic activity of a distinct PC within tumour samples has not been reported yet. Nevertheless, increased expression of PCs has been reported in various cancer types and was often found to correlate with aggressiveness of the malignancy.

Furthermore, based on the numerous substrates that are cleaved and activated by PCs, they may directly or indirectly influence the malignant phenotype and the potential for metastasis of cancer cells. Thus, they may modulate central processes like proliferation, survival, invasion and migration. Amongst the critical substrates involved in neoplasia formation and progression are growth factors and their receptors, matrix metalloproteases

(MMPs) and adhesion molecules. Examples of substrates will be discussed below; however the implication of furin activity in the formation and progression of different cancer types will be discussed in detail in section 5.2.

Proprotein convertases are highly expressed in certain cancers

The two neuroendocrine PCs, PC1/3 and PC2, were shown to be expressed in cancers composed of neural and/or endocrine cells (Jin, Kulig et al. 1999; Kajiwara, Itoh et al. 1999). For instance, high PC1/3 and PC2 expression was found in pheochromocytoma, neuroendocrine tumours of the medulla or adrenal glands, but was absent in normal adrenal tissue (Konoshita, Gasc et al. 1994). Furthermore, their expression pattern was correlated with the expression of their substrate proenkephalin (Breslin, Lindberg et al. 1993), suggesting function of PC1/3 and PC2 activity in tumours of the endocrine glands.

Aberrant PACE4 expression has been reported in breast cancer (Cheng, Watson et al. 1997). Furthermore, D'Anjou et al. found high expression of PACE4 in prostate cancers of all different clinical stages and showed, in functional studies, that decreased PACE levels in prostate cancer cell lines leads to reduced proliferation and clonogenic potential, and abrogates tumour growth in athymic nude mice (D'Anjou, Routhier et al. 2011). They further developed peptide-based inhibitors against PACE4 for treatment of prostate cancer, as discussed in section 4.6.

Studies examining PC5 and PC7 expression in different cancer entities are rather rare and the prognostic value of their expression is still elusive. Nevertheless, PC5 is expressed in colon cancer cell lines (Rovere, Barbero et al. 1998) and considerable PC7 expression was found in the majority of investigated breast cancer cell lines and tissues (Cheng, Watson et al. 1997).

Processing of growth factors and their receptors

Many cancer types are known to depend on growth factor signalling pathways for maintaining certain malignant characteristics such as unrestricted proliferation and high invasive potential. Various growth factors and their receptors involved in these processes require proteolytic activation by PCs to be converted into their mature forms. For instance IGF1R as well as its ligand IGF1 are processed by furin-like PCs (Duguay, Milewski et al. 1997; Khatib, Siegfried et al. 2001). Likewise, the activity of furin, PC5B or PACE4 seems to be necessary to process the precursor of the neurotrophin nerve growth factor (NGF) (Bresnahan, Leduc et al. 1990; Seidah, Benjannet et al. 1996). PCs also activate other growth factors whose functions have been linked to cancer progression, e.g. endothelin (Denault, Claing et al. 1995), parathyroid hormone (PTH) (Hendy, Bennett et al. 1995) and transforming

growth factor β1 (TGFβ1) (Dubois, Laprise et al. 1995). Siegfried et al. further characterised the catalytic events leading to activation of the platelet-derived growth factors (PDGF) A and B, a prerequisite for the role of these growth factors in tumourigenesis (Siegfried, Khatib et al. 2003; Siegfried, Basak et al. 2005). Additionally, PCs have been shown to be responsible for processing of the vascular endothelial growth factors (VEGF) C and D, growth factors that promote angiogenesis and lymphangiogenesis, thereby facilitating tumour growth and metastatic spread (Siegfried, Basak et al. 2003; McColl, Paavonen et al. 2007). In addition to IGF1R, other receptor tyrosine kinases are equally processed by PCs. Thus, the insulin receptor was reported to require PC-mediated processing in order to exert its function in glucose homeostasis (Robertson, Moehring et al. 1993; Hwang, Hernandez et al. 2000). In contrast, endoproteolytic cleavage of HGF by furin appears to be dispensable for its function (Komada, Hatsuzawa et al. 1993).

Matrix metalloproteases - destructors of the extracellular matrix

In order to successfully form metastasis a tumour cell proceeds through four essential steps: detachment, migration, invasion and adhesion. Thus, a metastasising cell has to be able to destruct the basement membrane and the surrounding ECM to escape the primary tumour. The ECM consists of various components, including collagen, gelatin, proteoglycans, laminin, fibronectin and vitronectin. Degradation of these components is mediated by proteases that are secreted by the malignant cells, but also by stroma cells. One of the main groups of ECM-degrading proteases is the family of matrix metalloproteases (MMPs). Under physiological conditions these enzymes with endopeptidase activity are tightly regulated and their expression is generally induced during wound healing or inflammation (reviewed in (Chakraborti, Mandal et al. 2003)). Nevertheless, high expression and activity of MMPs has been reported for various types of cancer and has been correlated with poor prognosis (reviewed in (Basset, Okada et al. 1997; Brown and Murray 2015)). Many MMPs and membrane-type MMPs (MT-MMPs) contain potential PC cleavage sites, however only a few of them have been shown experimentally to be PC substrates. One well studied example is MT1-MMP, also called MMP14, the best known member of the membrane-type subfamily of MMPs. The MT1-MMP zymogen may be cleaved by furin or PC5 (Maquoi, Noel et al. 1998; Yana and Weiss 2000). Active MT1-MMP in turn can directly cleave ECM components like collagen 1, but equally activates pro-MMP2 (Sato, Takino et al. 1994; Ohuchi, Imai et al. 1997). Other ECM-degrading metalloproteases processed by PCs include stromelysin-3 (Pei and Weiss 1995; Santavicca, Noel et al. 1996), the adamalysin metalloproteases (also a disintegrin and metalloprotease; ADAMs) (Loechel, Gilpin et al. 1998) and the adamalysin metalloproteases with thrombospondin motif (ADAM-TS) (Kuno, Terashima et al. 1999). Accordingly, application

of PC inhibitors was found to significantly lower the invasive potential of various tumour cells due to altered processing and activation of MMPs (reviewed in (Bassi, Fu et al. 2005)).

Cell adhesion molecules - important factors in metastasis

Cell adhesion molecules (CAMs) are proteins that are present on the cell surface and mediate cell-cell or cell-matrix interactions. Furthermore, they are involved in the activation of cell survival and proliferation mechanisms through the action of integrins. In the context of metastasis formation they play a pivotal role in migration and invasion of a tumour cell through lymphatic or angiogenic vessels as well as in arrest and extravasation of the cell at a distal site. Cell adhesion molecules can be grouped into three main families: immunoglobulins, integrins and selectins.

In the case of immunoglobulins, the implication of PCs is rather indirect as none of them seem to be direct substrates of PC processing. However, some of the cytokines and growth factors, like IGF1 or endothelins, which promote ICAM-1 or VCAM-1 expression in endothelial cells, are known PC substrates (Denault, Claing et al. 1995; Duguay, Lai-Zhang et al. 1995; Balaram, Agrawal et al. 1999; Ishizuka, Takamizawa-Matsumoto et al. 1999).

The importance of integrins in tumour progression and metastasis has been well established. Besides their role in adhesion of a cell to the ECM, integrins can control MMPs on the cell surface, interact with the cytoskeleton for alterations of cell shape and motility or transmit growth and survival signals (reviewed in (Seguin, Desgrosellier et al. 2015)). Integrins are heterodimeric receptors composed of an α- and a β-subunit. To date 18 α-subunits and eight β-subunits are known that form at least 24 different functional integrin receptors. This flexibility in formation of functional integrin receptors allows recognition of and adaptation to distinct environments. Half of the known α-subunits contain potential PC processing sites and α3, α4, α5, α6 and αv have been shown to be cleaved by furin, PC5 and to a lesser extent by PACE4 (Lehmann, Rigot et al. 1996; Lissitzky, Luis et al. 2000).

Selectins bind fucosylated carbohydrates, like sialyl-Lewis^{X} and sialyl-Lewis^{a}, and are comprised of the three family members E-selectin (endothelium), P-selectin (platelets) and L-selectin (lymphocytes), named after the cell type on which they were originally identified. They mediate adhesive interactions of leukocytes and platelets with the endothelium within the blood circulation, therefore similarities to dissemination of circulating tumour cells during metastasis have been hypothesised (Kannagi 1997; Krause and Turner 1999). Furthermore, as most cancer cells express increased levels of selectin ligands, which is associated with a poor prognosis, cell-cell interaction with selectins on leukocytes, platelets and endothelial cells was proposed to be required for metastatic progression (reviewed in (Laubli and Borsig 2010)). For instance, E-selectin appears to be a main homing receptor of breast and colon

cancer cells on activated endothelial cells (Tözeren, Kleinman et al. 1995) and adhesion of colon cancer cells to endothelial cell surface E-selectin seemed to be dependent on the amount of tumour cell-bound sialyl-Lewis^X, one of the E-selectin ligands (Izumi, Taniuchi et al. 1995). Additionally, tumour cells that enter the hepatic circulation seem to be able to trigger E-selectin expression on endothelial cells, thereby favouring extravasation to liver tissue (Khatib, Kontogiannea et al. 1999). E-selectin expression was shown to be induced by various cytokines, like IL-1 and TNFα, as well as by several growth factors including VEGF and IGF-1 (Kim, Moon et al. 2001; Che, Lerner-Marmarosh et al. 2002; Simiantonaki, Jayasinghe et al. 2002). Thus, although PCs do not directly process selectins, processing of cytokines and growth factors that might induce selectin expression indirectly favours formation of metastases.

4.6. Inhibitors of proprotein convertases

The involvement of PCs in many physiological and pathological processes has resulted in the development of many inhibitors. However, designing specific and efficient inhibitors remains a challenge, particularly for inhibition of the seven core members of this enzyme family.

One central issue is the lack of specificity of most PC antagonists caused by the common recognition site shared between furin-like PCs. Thus, many efforts have been made in order to determine the cleavage preference of distinct PCs. For instance, Remacle et al. used over 100 decapeptide sequences representing the R-X-(R/K/X)-R↓ motif from human, bacterial and viral proteins and evaluated the relative cleavage efficiency of furin, PC2, PC4, PC5, PACE4 and PC7 (Remacle, Shiryaev et al. 2008). Alas, they limited their analyses to known PC substrates and did not comment on the usefulness of their data for future inhibitor design. The work of others has been more concentrated on developing a tool to predict cleavage sites in potential PC substrates. Hence, Duckert et al. constructed artificial neural networks implementing furin- or PC-specific datasets in order to predict potential substrates (Duckert, Brunak et al. 2004). Despite these efforts, the information on cleavage preferences of distinct PCs are still sparse and design of specific inhibitors remains challenging. Nevertheless, the first furin specific inhibitors in the form of camelide antibodies, called nanobodies, have been recently described (Zhu, Declercq et al. 2012) and will be discussed in section 5.3.

Another important aspect that will be of concern for the clinical use of wide range PC inhibitors is the potential interference with the physiological function of these proteases. Nevertheless, it can be speculated that the redundancy in PC functionality that arises from

overlapping substrate preferences and intracellular or tissue expression might counteract most side effects.

Naturally occurring inhibitors of the neuroendocrine proprotein convertases

Among the endogenous inhibitors of PCs are 7B2 and ProSaas as well as the prosegments of the PCs, which will be discussed further below. As mentioned in the section about PC activation, 7B2 acts as a chaperone for proPC2 maturation and activity (Braks and Martens 1994). Within the ER, pro7B2 forms a complex with proPC2 and can be cleaved by PCs in the less alkaline, high calcium environment of the TGN. The released C-terminal part of processed 7B2 acts as an inhibitor of PC2. Once proPC2 is autocatalytically activated in the acidic environment of the secretory granules, mature PC2 as well as carboypeptidase E rapidly degrade 7B2 and the prosegment of PC2 (reviewed in (Khatib 2013)). In 1998, Apletalina et al. performed a positional scanning of a synthetic peptide combinatorial library to evaluate the inhibitory efficiency of hexapeptides on PC1/3 and PC2 (Apletalina, Appel et al. 1998). The hexapeptide LLRVKR proved to be the most potent inhibitor for both neuroendocrine PCs. This sequence represents the inhibitory sequence of proSAAS, a protein that is primarily found in the brain and other neuroendocrine tissue and that is generally cleaved by carboxypeptidase E (Fricker, McKinzie et al. 2000). Other than the hexapeptide alone proSAAS appears to be a specific inhibitor of PC1/3. ProSAAS and 7B2 exhibit some structural and functional homology: for instance both molecules contain a proline-rich sequence and a C-terminal peptide that can be cleaved by furin to exert the described inhibitory function (Cameron, Fortenberry et al. 2000). Thus, Cameron et al. proposed that proSAAS and 7B2 may belong to a new group of functionally homologous convertase inhibitors (Cameron, Fortenberry et al. 2000).

Prosegments of the proprotein convertases

Beside their chaperone function in guiding correct folding and intracellular trafficking, the prosegments of PCs also act as endogenous inhibitors of PCs. Interestingly, the inhibitory potency of furin-like PC prosegments was found to go beyond activity inhibition of the parent enzyme and various studies have demonstrated that all prosegments of furin-like PCs can act on various PCs (reviewed in (Khatib 2013)). Zhong et al. were the first to report the inhibitory potential of *ex vivo* expressed furin and PC7 prosegments on the processing of selected PC substrates including NGF (Zhong, Munzer et al. 1999). Soon thereafter, others confirmed the potent inhibitory function of the prosegments (Fugere, Limperis et al. 2002; Nour, Basak et al. 2003). Simultaneously, the potency of shorter peptides derived from the original prosegments was evaluated. Generally, complete prosegments seem to be more potent than shorter

peptides, whereby both display only limited specificity towards their cognate enzymes (Fugere, Limperis et al. 2002; Nour, Basak et al. 2003). Nevertheless, Bhattacharjya et al. identified a 24 amino acid long peptide derived from the C-terminal end of the PC7 prosegment that showed comparable inhibition of PC7 as compared to the full length fragment (Bhattacharjya, Xu et al. 2000). Consequently, expression of PC prosegments has often been used as a tool to demonstrate the involvement of PC activity in the processing of suspected substrates, e.g. VEGF-C, PDGF-A and PDGF-B (Siegfried, Basak et al. 2003; Siegfried, Khatib et al. 2003; Siegfried, Basak et al. 2005).

Small molecule- and peptide-based inhibitors

Expression of the pan-PC inhibitor α1-antitrypsine Portland, mostly referred to as α1-PDX, is one of the most common tools of research to investigate the effect of furin-like PC inhibition on the behaviour of cells. The serine protease inhibitor (serpin) was derived from serial mutations of α1-antitrypsine which shifted the specificity of inhibition from the neutrophil elastase, to PCs (Owen, Brennan et al. 1983; Anderson, Thomas et al. 1993; Wright 1996). Its potential of inhibiting PCs was shown by Anderson et al. and Benjannet et al. (Anderson, Thomas et al. 1993; Benjannet, Savaria et al. 1997). Further modifications led to two variants, RRRRSA and AVNR, that appear to be highly selective for PACE4 and PC5 or furin, respectively (Tsuji, Kanie et al. 2007; Hada, Isshiki et al. 2013). Like other serpins, α1-PDX is an inhibitor that undergoes conformational changes upon cleavage leading to the formation of a stable enzyme-inhibitor complex (Wright 1996; Tsuji, Kanie et al. 2007). Experimentally α1-PDX is mostly introduced into cells by expression of recombinant protein; however purified recombinant α1-PDX became commercially available very recently and has been used for instance on osteosarcoma cell lines (Liu, Li et al. 2014).

Other small-molecule inhibitors are cell-permeable and act mostly in a competitive manner, e.g. dicoumarol derivates (Komiyama, Coppola et al. 2009), 2,5-dideoxystreptamine derivates (Jiao, Cregar et al. 2006) and decarboxylated P1 arginine peptide mimetics (Becker, Sielaff et al. 2010). Incorporation of peptide motifs consisting of patterns of basic amino acid residues has been explored by many groups, resulting, among others, in the description of a nona-D-arginine peptide (Kacprzak, Peinado et al. 2004). Similarly, the compound decanoyl-RVKR-chloromethylketone, mostly referred to as dec-CMK or CMK, has been often used as an irreversible pan-PC inhibitor in experimental setups (Garten, Stieneke et al. 1989; Denault, Claing et al. 1995; de Cicco, Bassi et al. 2007).

PCSK9 inhibitors

The most promising PC inhibitors to date are the ones that target active PCSK9 to treat hypercholesterolaemia and prevent coronary artery disease due to PCSK9's unique role in protein degradation of the low density lipoprotein receptor (LDLR) (Seidah, Benjannet et al. 2003; Chan, Piper et al. 2009; Seidah and Prat 2012). Several approaches have been investigated to decrease the amounts of circulating PCSK9 or levels of PCSK9 mRNA in hepatocytes, which are the major source of circulating PCSK9. Small-molecule inhibitors could be administered orally and either interfere allosterically with the PCSK9-LDLR interaction or interrupt the autocatalytic processing of PCSK9 in the ER. The company Serometrix has adopted the first approach with their compound SX-PCSK9, whereas two other companies, Zydus Cadila group and Shifa Biomedical Corporation, opted for the latter option. Nevertheless, information on specific compounds and clinical advances are sparse (reviewed in (Seidah 2013)). Furthermore, Bristol-Myers Squibb/Adnexus described fibronectin derived adnectins. These around 12 kDa molecules are cheap to produce, but have a rather short plasma half life due to quick kidney clearance (discussed in (Seidah 2013)). Application of antisense RNAi targeting PCSK9, such as the lipoid nanoparticle formulation ALN-PCS from the company Alnylam, aims at reducing PCSK9 mRNA levels in hepatocytes and seems promising (company website and (Frank-Kamenetsky, Grefhorst et al. 2008)).

Several monoclonal antibodies have been developed to interrupt the interaction of PCSK9 and LDLR (Chan, Piper et al. 2009; Ni, Di Marco et al. 2011; Liang, Chaparro-Riggers et al. 2012). A meta-analysis of phase II and III trials recently compared 24 studies and confirmed the potential of PCSK9 antibodies to reduce LDL cholesterol levels and predicted safety and efficacy (Navarese, Kolodziejczak et al. 2015). In general, inhibition of PCSK9 is hypothesised to be more specific and have fewer side effects than the use of statins, but might also be used in combination with statins to counteract their PCSK9 upregulating function that attenuates the decrease in LDL cholesterol (Rashid, Curtis et al. 2005; Marais, Blom et al. 2012; Seidah and Prat 2012). Overall, anti-sense RNA, adnectins and small-molecule inhibitors of PCSK9 are mostly in pre-clinical testing phases or entered phase I clinical trials, whereas the application of different monoclonal antibodies against PCSK9 is more advanced and phase III trials have commenced with >20,000 enrolled individuals (reviewed in (Seidah 2013)).

5. Furin

Furin was identified as the first member of the PC family over 25 years ago (van de Ven, Voorberg et al. 1990). Due to its ubiquitousness and biological importance, furin is also the most studied member of this enzyme family. Some of its most important characteristics are summarised in Table 3.

Table 3 Furin at a glance.

Chromosomal location	Mouse 7D1-E2	Human 15q26.1
Tissue distribution	Ubiquitious	
Subcellular localisation	TGN, cell surface, endosomes	
Secretion	Shed	
Amino acids	794	
Predicted molecular weight	pro-furin 86.7 kDa	mature furin 74.3 kDa
Prosegment sequence	YHFWH***RGVTKR***SLSPHRPRHSRLQREPQVQWLEQQVA***KRRTKR***DVY	
Internal cleavage sites	First KRRTKR^{107}↓	Second RGVTKR^{75}↓
Consensus cleavage site	R-X-R/K-R	
pH optimum	5-8 (substrate dependent)	
Calcium requirement	Around 1 mM	
Functional redundancy with other PCs	Strong redundancy with PACE4 and PC5, partial redundancy with PC7	

PACE4, paired basic amino acid cleaving enzyme 4; PC, proprotein convertase; TGN, trans-Golgi network. Chromosomal locations: National Center for Biotechnology Information (NCBI); mouse furin gene ID- 18550; human furin gene ID- 5045. Molecular weight prediction: ExPASy, Compute pI/MW tool (Gasteiger, Hoogland et al. 2005)

5.1. The proprotein convertase furin at a glance

From gene to active enzyme

The locus of the *FURIN* gene, also called *FUR* (fes/fps upstream region) gene, was first described by Roebroek et al. in 1986 (Roebroek, Schalken et al. 1986; Roebroek, Schalken et al. 1986a). It lays in the upstream region of the c-fes/fps proto-oncogene, a gene that encodes the protein tyrosine kinase c-Fes which is involved in myeloid cell development (reviewed in (Kim, Ogata et al. 2004) and has been associated, among others, with prostate cancer recurrence (Miyata, Watanabe et al. 2012). The *FURIN* gene is located on chromosome 15 in position q26.1 (NCBI, gene ID: 5045). Interestingly, *PCSK6*, the gene of PACE4, is located at 15q26.3 (NCBI, gene ID: 5046) and is thus situated in close proximity to the *FURIN* gene, suggesting that both evolved from a common ancestor (Kiefer, Tucker et al. 1991). The loci of the other human *PCSK* genes are spread throughout the genome.

A few years after the identification of the *FURIN* gene, Fuller et al. and van de Ven et al. characterised the associated protein furin as the mammalian homologue to the prohormone processing yeast kexin (Fuller, Brake et al. 1989; van de Ven, Voorberg et al. 1990). Furin is translated as a 794 aa zymogen that undergoes several posttranslational modifications to give rise to the active type-1 transmembrane protein (reviewed in (Thomas 2002)). Firstly, the signal peptide that directs localisation to the ER is removed through signal peptidase-mediated cleavage. As discussed in section 4.2., furin undergoes a two-step, compartment-specific autocatalytic cleavage of its 83 aa prosegment. The first cleavage step is rather fast with a half life of $t_{1/2}$=10 min and occurs at Arg^{107} in the ER. Once the prosegment-furin complex trafficked to the mildly acidic environment of the TGN/endosomal system, the prosegment is cleaved a second time. This cleavage is pH sensitive, rather slow with a half life of $t_{1/2}$<2h and processes the prosegment at Arg^{75}, leading to the liberation of the inhibitory prosegment. However, furin requires a specific calcium concentration and pH for optimal activity, which will be discussed further below. Another reported posttranslational modification is the phosphorylation of the cytoplasmic tail through casein kinase 2, regulating intracellular localisation of furin (Jones, Thomas et al. 1995). Three potential *N*-linked glycosylation sites, Asn^{387}, Asn^{440} and Asn^{553}, have been identified; however, reports on their importance in maintaining furin activity are conflicting (Creemers, Vey et al. 1995; McCulloch, Wylie et al. 2010).

Subcellular localisation and tissue distribution

Furin orthologues are found in all vertebrates and many invertebrates including model organisms such as mouse, rat or *Drosophila melanogaster* (Seidah, Day et al. 1998). As PC7, furin appears to be ubiquitously expressed, whereby expression levels between different tissues may vary. Higher quantities of furin are found in the human liver and kidney (Seidah, Day et al. 1998). Similarly, furin, as well as PC7 expression have been confirmed in all investigated cell lines (The Human Protein Atlas: Furin, CAB009499).

Furin cleaves its substrates within the constitutive secretory pathway. Accordingly, mature furin can be found in the TGN, on the cell surface, as well as in endosomes where it may cleave its substrates (reviewed in (Molloy, Anderson et al. 1999)). The majority of furin appears to be located in the TGN (Bosshart, Humphrey et al. 1994), from where it cycles to endosomes, the cell surface and back. From the TGN, furin is sorted to endosomes via clathrin coated vesicles, a process that is dependent on the recruitment of AP-1 (adaptor protein 1) (Teuchert, Schäfer et al. 1999). Furin tethering to the cell surface, on the other hand, seems to be mediated through interaction with filamin ABP-280 (Liu, Thomas et al. 1997). Endocytosis of cell surface furin is initiated through binding of furin to the μ2 subunit of plasma membrane-specific AP-2 (Teuchert, Berghöfer et al. 1999). Whether endosomal furin

is then recycled back to the cell surface or redirect to the TGN appears to be, at least in part, dependent on the phosphorylation of its cytoplasmic tail through casein kinase 2. Interestingly, phosphorylation of the acidic cluster and interaction with the sorting protein PACS-1 (phosphofurin acidic cluster sorting protein-1) favour recycling to the cell surface, but are equally required for retrieval of furin from endosomes to the TGN (Wan, Molloy et al. 1998; Crump, Xiang et al. 2001). Thus, dephosphorylation of the cytoplasmic tail by specific isoforms of protein phosphatase 2A (PP2A), which was proposed to occur prior to the transit to late endosomes, liberates furin from the phosphorylation-dependent local cycling loop between early endosomes and the cell surface, thereby enabling sorting to the TGN (Molloy, Thomas et al. 1998). Degradation of furin occurs in lysosomes with a proposed half life of $t_{1/2}$=2-4h (Bosshart, Humphrey et al. 1994). Aggregation of furin in the TGN is likely mediated via its cytoplasmic domain and might trigger delivery to lysosomal compartments for subsequent degradation (Wolins, Bosshart et al. 1997).

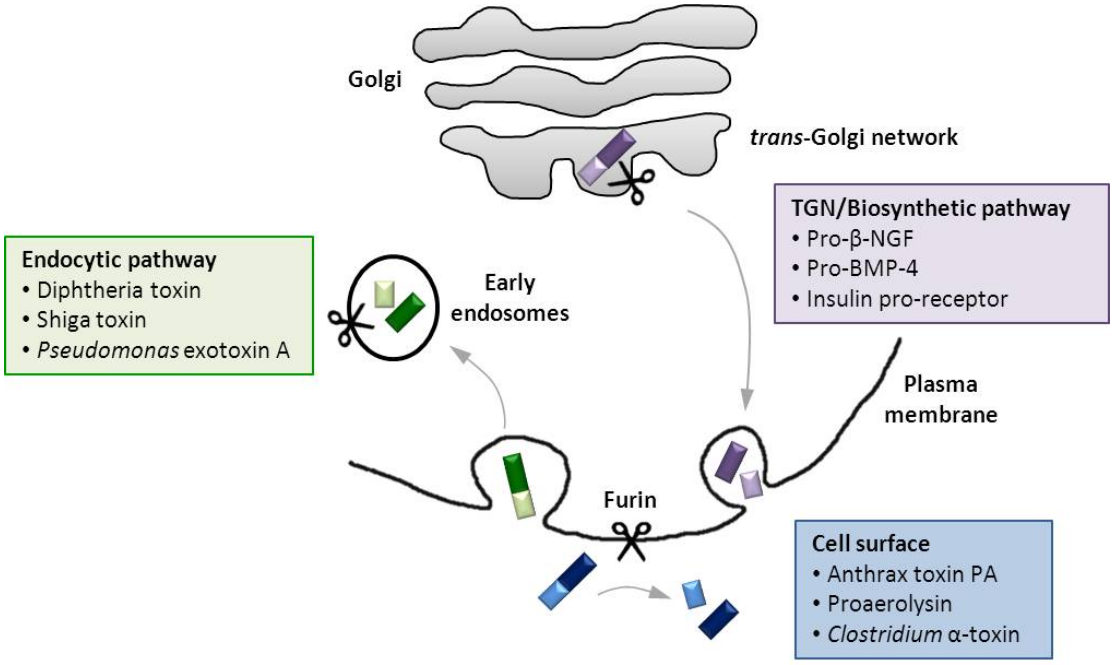

Figure 5 Compartments of furin substrate processing within the *trans*-Golgi network/endosomal system.
At steady state, furin (represented by scissors) is predominantly localised to the *trans*-Golgi network (TGN), from where it cycles to the cell surface, endosomes and back. In the TGN/biosynthetic pathway, furin processes many secreted prohormones like pro-β-nerve growth factor (pro-β-NGF) or pro-bone morphogenic protein-4 (pro-BMP-4). Cell surface furin mainly cleaves extracellular substrates such as anthrax toxin protective antigen (PA). Other bacterial toxins, e.g. diphtheria toxin and shiga toxin, are preferably cleaved in mildly acidic early endosomes within the endocytic pathway. *Modified from (Thomas 2002).*

Domain structure of furin

The general domain structure of furin follows that of the other furin-like PCs (Figure 3 and Figure 6). The N-terminal part comprises four important domains: 1) signal peptide directing translocation to the ER during translation, 2) prosegment with chaperone function, 3) highly conserved catalytic domain and 4) P domain. The latter regulates the stability, calcium and pH dependence of furin (Zhou, Martin et al. 1998). The P domain also contains a conserved RGD motif, whose function is not well understood. The more individual C-terminal part of furin contains a Cys-rich domain, a transmembrane domain and a short cytoplasmic tail. The cytoplasmic tail was shown to be both necessary and sufficient to direct TGN localisation of mature furin (Bosshart, Humphrey et al. 1994; Chapman and Munro 1994; Molloy, Thomas et al. 1994). More precisely, TGN localisation was found to depend on the presence of an acidic amino acid cluster, CPSDSEEDEG^{783}, and phosphorylation of serine residues by casein kinase 2 (Jones, Thomas et al. 1995; Schäfer, Stroh et al. 1995; Voorhees, Deignan et al. 1995). Recruitment of AP-1 and interaction with its µ1 subunit, a prerequisite for vesicle formation upon budding from the TGN, is mediated by a tyrosine tetrapeptide motif YKGL^{765} and to a lesser extent by a leucine-isoleucine signal LI^{760} (Teuchert, Schäfer et al. 1999). Furthermore, YKGL^{765}, LI^{760} as well as F^{790} constitute important motifs for recycling of cell surface furin by mediating sorting or tethering to early endosomes (Jones, Thomas et al. 1995; Voorhees, Deignan et al. 1995; Teuchert, Berghöfer et al. 1999).

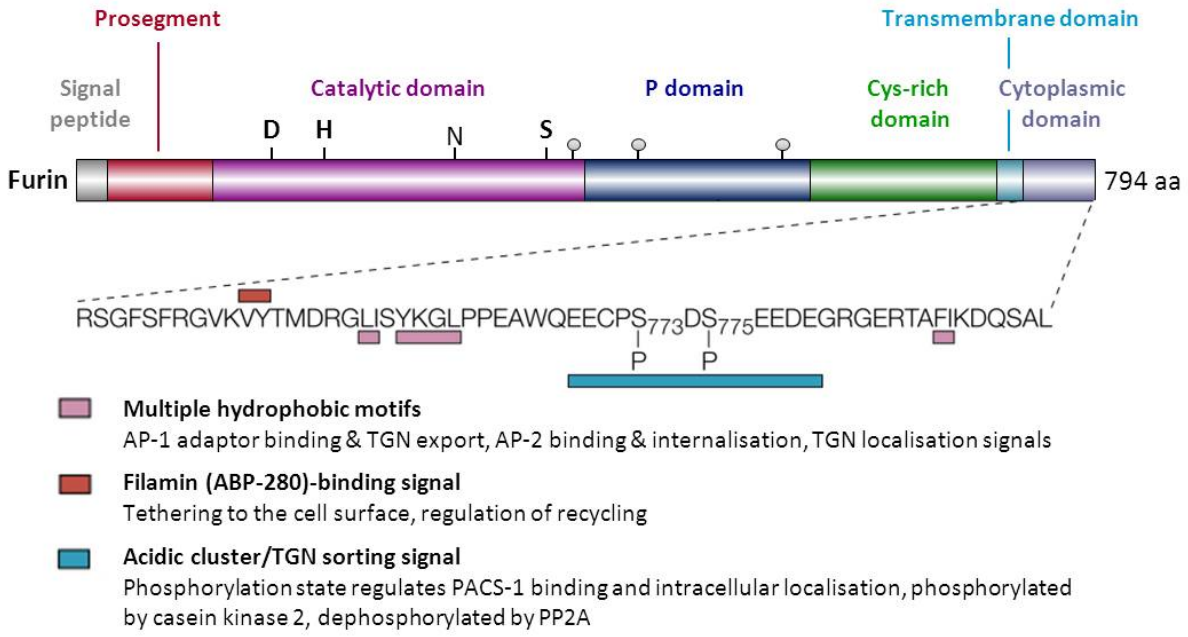

Figure 6 The furin sorting motifs of the cytoplasmic domain.
Shown is the primary structure of human furin with a focus on its cytoplasmic domain and its contained intracellular sorting motifs. AP, adaptor protein; PACS-1, phosphofurin acidic cluster sorting protein-1; PP2A, protein phosphatase 2A; TGN, trans-Golgi network. *Modified from (Thomas 2002).*

Cleavage preference and active site

Many efforts have been made to elucidate the stringent cleavage preference of furin-like PCs for basic amino acid motifs and to uncover possible differences between the PCs in order to design specific inhibitors. An important step was done in 2003 when Henrich et al. described the first crystal structure of the mouse furin ectodomain, amino acids 108-582 (Henrich, Cameron et al. 2003). This 2.6 Å crystal structure comprises the catalytic domain, bound to the pan-PC inhibitor CMK, as well as the P domain (Figure 7A). The obtained crystals contained eight molecules per asymmetric unit, each consisting of two separate domains, the spherical catalytic domain (amino acids 110-445) and the barrel-like P domain (amino acids 446-573). The core of the catalytic domain basically consists of a twisted β-sheet and in total seven flanking α-helices connected by two β-hairpin loops. Some extended surface loops were shown to mediate interactions with the P domain or shape the active site cleft. Furthermore, two internal disulfide bridges and two calcium binding sites were identified. The glycosylation state of the three potential N-linked glycosylation sites could be determined, whereby Asn^{387} seemed to be monoglycosylated and Asn^{440} bound up to eleven sugars. The P domain on the other hand is organised as an eight-stranded β-sandwich similar to a jelly-roll β-barrel. The remaining C-terminus of furin, including the transmembrane domain, was predicted to consist of unstructured regions. Based on the interactions of the catalytic domain and the P domain, the latter was proposed to exert a stabilising function by shielding the catalytic domain from the bulk solvent, potentially preventing aggregation.

The active site of furin resembles a crevice with the catalytic triad Ser^{368}-His^{194}-Asp^{153} arranged in its centre (Figure 7B). The preference for the stringent consensus cleavage sequence R-X-K/R-R↓ of furin was elucidated on the basis of the covalently bound inhibitor CMK that harbours the tetrapeptide RVKR. The P1-Arg side chain spreads into the S1 pocket that would fit any other side chain, including that of lysine, less well due to its specific structure. The charges and the geometry of the S2 pocket on the other hand benefit a P2-Lys side chain, but would also allow an Arg side chain. The P3-Val side chain points into the bulk solvent, thus explaining the relative lack of amino acid preference. The cleft that accommodates the P4 residue favours an Arg or to a lesser extent a Lys side chain, but is not exclusive for those two residues. Therefore, a short P4 residue can be compensated for by a favourable P6 residue whose side chain might extend into the S4 pocket, as it is the case with the second autocatalytic cleavage site $RGVTKR^{75}$↓ within the prosegment.

In conclusion, this report confirms previous hypotheses and explains the preference for multiple basic residues in the cleavage sequence based on electrostatic interactions between the negatively charged catalytic domain and the substrate (van de Ven, Voorberg et al. 1990; Siezen, Creemers et al. 1994). Further structural investigations comparing the crystal

structures of mouse furin (Henrich, Cameron et al. 2003) and Kex2, both bound to covalent inhibitors, showed the similarities of the catalytic domains, both in overall fold and topology. These comparisons further explained, based on structural differences, the stronger basic amino acid preferences in P2 and P4 for Kex2 and furin, respectively (Holyoak, Kettner et al. 2004; Rockwell and Thorner 2004). Crystallisation of human furin in complex with noncovalent inhibitors highlighted the high overall structural similarities of the mouse and human catalytic and P domains, and supports previously reported amino acid preferences (Dahms, Hardes et al. 2014).

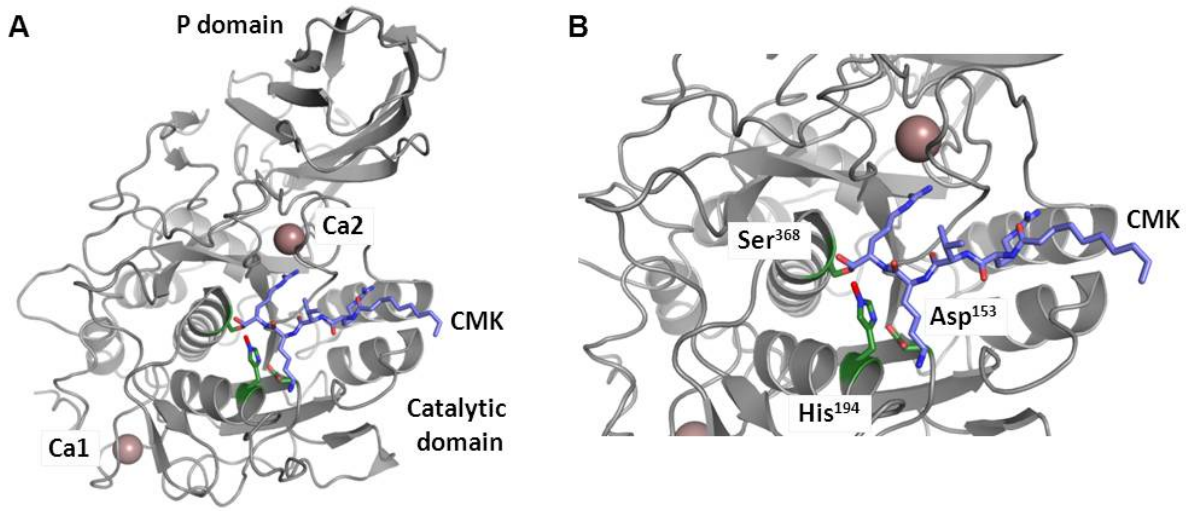

Figure 7 Three dimensional structure of the CMK-inhibited mouse furin ectodomain.
A) Stereo ribbon plot of soluble furin. The spherical catalytic domain (amino acids 110-445) contains two calcium ion binding sites (Ca1 and Ca2) and has a CMK molecule bound in its active site. The P domain (amino acids 446-573) is of barrel-like shape. B) Detailed view of the active site. The inhibitor CMK (blue stick model) is covalently bound via its hemiketal methyl group to the Oγ of Ser^{368}. The three residues of the catalytic triad, Ser^{368}, His^{194} and Asp^{153}, are depicted in green.
PDB entry: 1P8J, pictures prepared with the programme PyMOL (Schrodinger 2015).

Calcium and pH dependence

The calcium ion dependence of furin for furin substrate processing has been shown *in vitro* (Bresnahan, Leduc et al. 1990; Molloy, Bresnahan et al. 1992; Stieneke-Grober, Vey et al. 1992). Furthermore, autocatalytic activation of furin is dependent on the presence of calcium ions, whereby maximal activity appears to require a millimolar range calcium concentration (Vey, Schafer et al. 1994). This calcium dependence was explained by the crystal structure of mouse furin that showed that the calcium ion in the second binding site, Ca2, seems to stabilise the S1 pocket, thereby determining the preference for Arg in P1 (Henrich, Cameron et al. 2003). Further structural studies confirmed Ca2 to be essential for P1

specificity and pointed out that furin contains no K^+ binding sites (Than, Henrich et al. 2005). Crystallisation of the catalytic and P domains of human furin indicated the presence of a third calcium binding site, as well as a sodium binding site, however functional studies are elusive so far (Dahms, Hardes et al. 2014).

The pH dependence of furin has been described mostly for the two consecutive cleavage steps and removal of the furin prosegment. Anderson et al. were the first to investigate in more detail the second cleavage at $RGVTKR^{75}\downarrow$ that occurs in the mildly acidic and calcium containing environment of the TGN (Anderson, VanSlyke et al. 1997). They determined the pH optimum to be 6.0 and hypothesised that binding of two calcium ions in the proposed pockets is required for secondary cleavage of the prosegment. Nuclear magnetic resonance (NMR) studies a few years later showed that the pH most likely influences the conformational stability of the prosegment, thus the prosegment unfolds under acidic conditions leading to exposure of the secondary cleavage site (Bhattacharjya, Xu et al. 2001). Conserved His^{69}, which directly precedes the second internal cleavage site in the prosegment, was finally described to be an internal pH sensor (Feliciangeli, Thomas et al. 2006; Williamson, Elferich et al. 2013). Unprotonated His^{69} in the ER stabilises a hydrophobic pocket required for primary cleavage at Arg^{107}, whereby protonation of His^{69} in the TGN leads to disruption of this pocket and exposure of the cleavage site at Arg^{75}. Histidine protonation as a reason for furin prosegment unfolding was further confirmed by Dillon et al. who compared the pH dependence of the prosegments of furin and PC1/3 and attributed the higher pH optimum of furin to the 2-fold higher overall histidine content of the prosegment (Dillon, Williamson et al. 2012). Furthermore, His^{69} in PC1/3 appears to have an acid-shifted pK_a (acid dissociation constant) compared to furin, thus explaining the lower pH optimum (pH~5.5) of PC1/3 (Elferich, Williamson et al. 2015).

Furin knockout models and the role of furin in embryogenesis

As for most PCs, the physiological role of furin has been deduced from the use of knockout mice (reviewed in (Scamuffa, Calvo et al. 2006)). Zheng et al. investigated the expression pattern of furin and observed that it changes throughout rat development (Zheng, Streck et al. 1994). Between E7 and E10, furin was found to be expressed in the endoderm and mesoderm, whereby increased levels were observed in heart and liver primordia around E10. At later stages, furin appears to be broadly expressed in the peripheral tissues. The complete absence of furin in PC null mice leads to lethality in early embryonic stages, which is also seen for the phenotype of PC5 and SKI-1 null mice. Thus, embryos with lack of furin died between E10.5 and E11.5, presumably based on haemodynamic insufficiencies, ventral closure defects and failed axial rotation (Roebroek, Umans et al. 1998). An endothelial cell

specific knockout of furin in mice led to ventricular septal defects and/or valve malformations, was associated with disrupted endothelin-1 and bone morphogenetic protein 4 (BMP4) maturation in lung endothel cells, resulting in death shortly after birth (Kim, Essalmani et al. 2012). TGFβ is another furin substrate hypothesised to play a role in the embryonic cardiovascular system (Scamuffa, Calvo et al. 2006), as embryos deficient in TGFβ showed a similar phenotype to furin deficient embryos (reviewed in (Dunker and Krieglstein 2000).

Visualisation of furin-like activity

In order to gain a better understanding of the role of furin activity in embryogenesis or processes like tumour growth and migration it would be of great advantage to be able to visualise furin activity in a non-invasive manner. Furthermore, such methods would create the unique opportunity to directly assess efficacy of inhibitory drugs *in vivo*. One of the few reported approaches described the use of bioluminogenic furin probes based on tetrapeptides representing optimal furin cleavage motifs coupled to D-aminoluciferin, an analogue of D-luciferin (Dragulescu-Andrasi, Liang et al. 2009). Removal of the peptide group upon processing through furin generates free D-aminoluciferin, leading to bioluminescent signal emission in the presence of firefly luciferase. It was shown that these probes are cell permeable *in vitro*, lead to furin activity-dependent bioluminescence in breast cancer cells *in vitro* and could be used to visualise furin activity in breast cancer xenografts in living mice (Dragulescu-Andrasi, Liang et al. 2009).

Another group used the well known Förster resonance energy transfer (FRET) phenomenon to create probes that consist of two fluorophores joined by the PC recognition motif RQRR (Mesnard and Constam 2010). As cleavage of the non-cell permeable probes leads to proportional loss of FRET, they were able to follow the activity pattern of cell surface bound PCs during mouse embryo development. In contrast to the above described bioluminescence or fluorescence based imaging approaches, which suffer from strong scattering of light in deep biological tissues, photoacoustic imaging might provide higher sensitivity and simplicity in target validation in deep tissues. Thus, photoacoustic imaging, a combination of optical and ultrasound imaging technologies, offers high spatial resolution at a depth of several centimetres and is therefore highly suitable for biomedical imaging. In 2012, Liang et al. described a furin activity-driven condensation reaction between 1,2-aminothiol and 2-cyanobenzothiazole resulting in the formation of nanostructures, which was proposed for imaging of furin activity in cells (Liang, Ren et al. 2010). Dragulescu-Andrasi et al. redesigned the probe in order to generate an oligomerisable probe that accumulates at the target site upon furin processing and serves as an exogenous contrast agent for photoacoustic imaging (Dragulescu-Andrasi, Kothapalli et al. 2013).

Despite their great potential for visualisation of furin overexpressing tumours in patients, all described imaging probes appear to be rather furin-like PC specific than exclusively furin specific, as they rely on cleavage of tetrapeptide sequences which might be recognised by several furin-like PCs.

5.2. Furin and cancer

Tumourigenesis requires the activity of numerous proteins that support the malignant phenotype by maintaining tumour-related capabilities such as sustained proliferation, evasion of apoptosis, angiogenesis or activation of invasive and metastatic processes. Many of those cancer-related proteins are overexpressed and have to be proteolytically processed. Therefore, high activity levels of involved proteases likely favours cancer progression and metastasis. Accordingly, high expression of PCs has been found in various human tumours and tumour cell lines (see also section 4.5.).

Furin expression is associated with high aggressiveness in several carcinomas

High furin expression levels have been found in lung tumours and lung cancer cells. As furin expression appears to be elevated in non-small cell lung carcinomas (NSCLCs) it was proposed as a marker to discriminate between NSCLCs and small cell lung carcinomas (SCLCs)(Schalken, Roebroek et al. 1987). Low levels of furin mRNA in SCLCs were confirmed in a study by Mbikay et al. (Mbikay, Sirois et al. 1997). However, both studies are limited to expression levels and do not comment on the functional relationship between furin expression and the malignancy state of lung cancer types. Nevertheless, a recent study employing the inhibitor α1-PDX showed that inhibition of furin-like PCs might reduce proliferation and migration of the lung carcinoma cell line A549 (Ma, Fan et al. 2014).

Apart from lung carcinomas furin expression has also been associated with other carcinomas, whereby functional investigations remain elusive in most cases. Thus, furin and VEGF-C expression were examined in squamous cell carcinoma and it was proposed that furin might be a marker for tumour progression and potentially cleaves VEGF-C, leading to higher microvessel density (Lopez de Cicco, Watson et al. 2004). High levels of furin have also been found in ovarian cancer specimens and cell lines, whereby furin expression appeared to be linked to a poor outcome in patients (Page, Klein-Szanto et al. 2007). Furthermore, furin expression was confirmed in a small set of gastric cancer tissues and cell lines, where it was proposed to regulate maturation of the parathyroid hormone-related peptide (Nakajima, Konda et al. 2002). Overexpression of furin in a gastric cancer cell line further suggested a possible role of furin in gastric cancer growth.

In other cancer types, furin expression and activity have been studied in more detail. Cheng et al. investigated the expression of six PCs in breast cancer samples and stated that PC1/3, furin, PACE4 and PC7 mRNA levels are detected in most cases (Cheng, Watson et al. 1997). Furthermore, furin expression seemed to be confined to tumour cells as the surrounding tissue was furin negative. A follow-up study could further show that exogenous overexpression of either PC1/3 or furin not only increases the proliferation rate in the breast cancer cell line MCF7, but also conveys a certain level of anti-oestrogen resistance upon treatment with tamoxifen (Cheng, Xu et al. 2001). Additionally, Lapierre et al. used exogenous expression of furin and PACE4 prosegments to decipher their role in the highly malignant MDA-MB-231 breast cancer cell line and found that both PCs seem to exert opposing roles (Lapierre, Siegfried et al. 2007). Furin activity positively correlated with the aggressive phenotype and inhibition led to reduced cell motility, migration and invasion, whereas PACE4 inhibition even enhanced these features. This report shows that PCs may exert distinct functions in the same cancer or even act in an opposing manner. Thus, it would be of importance to assess expression levels of all related PCs upon investigation of a distinct PC in a certain tumour type.

Head and neck tumours represent an additional group of tumours that are associated with elevated levels of furin. Thus, increased furin mRNA and protein levels correlate with increased levels of maturated MT1-MMP and aggressiveness of head and neck squamous cell carcinomas (HNSCCs), as measured by presence of metastases in patients (Bassi, Mahloogi et al. 2001). Further investigations showed that inhibition of furin-like PCs through expression of α1-PDX reduces TGFβ, MT1-MMP and MMP2 processing and decreases the invasive potential of invasive HNSCC cell lines *in vitro* as well as *in vivo*. α1-PDX expression also clearly reduced tumourigenicity upon subcutaneous inoculation of modified cells (Bassi, Lopez De Cicco et al. 2001). Accordingly, overexpression of furin in HNSCC cell lines with moderate invasive potential increased MT1-MMP processing led to earlier tumour growth and enhanced invasiveness *in vitro* and *in vivo* (Bassi, Mahloogi et al. 2003). Hence, high furin activity in HNSCC cells favours metastatic and invasive potential most likely by mediating sustained processing and high activity of matrix degrading proteases like MT1-MMP and indirectly MMP2.

Furin substrates are implied in tumour progression and metastasis

Many of the proteins that were described to require processing by furin or furin-like PCs are involved in tumour progression and/or metastasis. As most of them have already been described in section 4.5, this section will focus on a few selected furin substrates and their implication in tumour formation and aggressiveness (Figure 8).

Many growth factors that have been implicated in sustained proliferation of tumour cells are activated by furin processing. These include PDGF-A and -B as well as TGFβ (Dubois, Laprise et al. 1995; Siegfried, Khatib et al. 2003; Siegfried, Basak et al. 2005). Furthermore, furin activity is required for active IGF signalling, as it matures the receptor tyrosine kinase IGF1R as well as its natural ligands IGF1 and 2 (Duguay, Lai-Zhang et al. 1995; Duguay, Jin et al. 1998; Khatib, Siegfried et al. 2001). Besides supporting proliferation, aberrant IGF signalling also favours anti-apoptotic effects, mediated by MAPK or PI3 kinase pathways, respectively (Gooch, Van Den Berg et al. 1999).

Furthermore, efficient tumour growth requires sufficient supply of oxygen and nutrients, highlighting the importance of adequate vascularisation. It could be shown that VEGF-C as well as VEGF-D have to be fully processed by furin or related PCs to stimulate VEGF signalling and induce angiogenesis and lymphangiogenesis (Millauer, Wizigmann-Voos et al. 1993; Joukov, Sorsa et al. 1997; McColl, Paavonen et al. 2007). Additionally, only mature VEGF-C appears to be able to initiate tumourigenesis in mice (Siegfried, Basak et al. 2003).

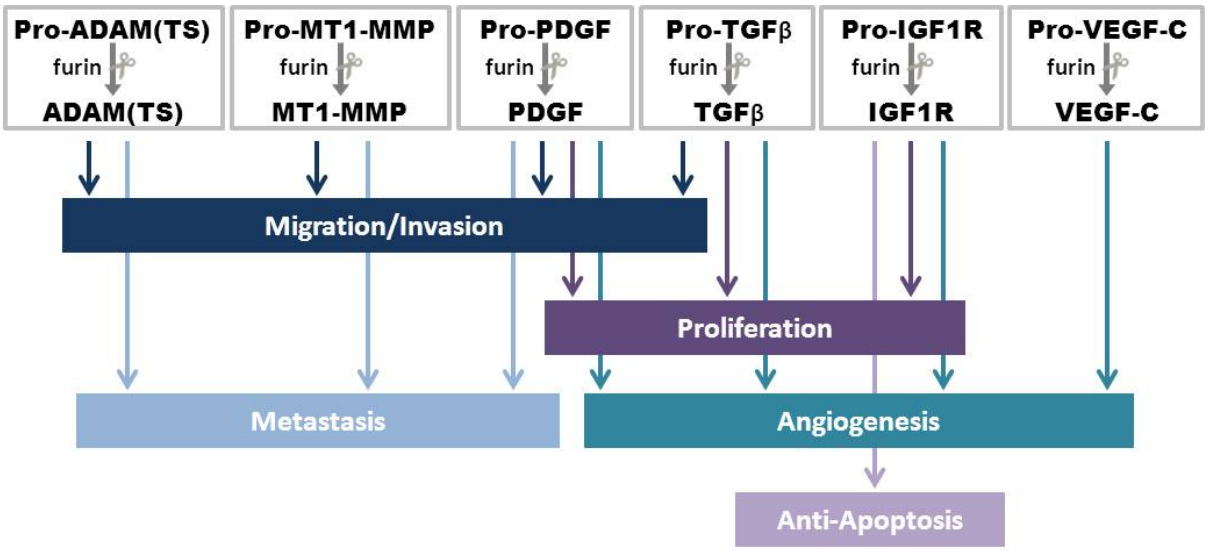

Figure 8 Substrates of furin and their involvement in tumour progression.
Schematic representation of some cancer-related proteins that are cleaved by furin and furin-like PCs and their relevance in important cancer-related processes. ADAM, a disintegrin and metalloprotease; IGF1R, insulin-like growth factor 1 receptor; MT1-MMP, membrane type 1 matrix metalloprotease; PDGF, platelet derived growth factor; TGFβ, transforming growth factor β; VEGF-C, vascular endothelial growth factor C. *Modified from (Bassi, Fu et al. 2005).*

. Some of the described substrates exert very diverse functions related to cancer progression. For instance, PDGF signalling is not only a potent mitogen, autocrine PDGF signalling equally promotes metastatic potential of breast cancer and pancreatic cancer cells, (Jechlinger, Sommer et al. 2006; Weissmueller, Manchado et al. 2014). In addition, PDGF-BB

appears to induce endothelial cell proliferation, migration and sprouting through paracrine stimulation of its receptor PDGFR-β, thereby favouring tumour angiogenesis (Xue, Lim et al. 2012).

Several other furin substrates could be shown to play a central role in dissemination, motility and metastasis formation of cancer cells. As mentioned previously, furin catalytically activates MT1-MMP (Maquoi, Noel et al. 1998; Yana and Weiss 2000; Remacle, Rozanov et al. 2006), which has been linked to progression of head and neck squamous cell carcinomas (Bassi, Lopez De Cicco et al. 2001; Bassi, Mahloogi et al. 2001). MT1-MMP in turn is the main enzyme to process pro-MMP2 on the cell surface (Sato, Takino et al. 1994; Ohuchi, Imai et al. 1997), resulting in an intermediate 64 kDa form that requires further intermolecular autocleavage to yield fully active 62 kDa MMP2, which is also called gelatinase A, a major mediator of basement membrane degradation (Atkinson, Crabbe et al. 1995; Strongin, Collier et al. 1995). Besides the well studied cell surface activation of MMP2, intracellular cleavage of the precursor protein has been reported (Lee, Akers et al. 1997) and further investigations suggested that intracellular processing of pro-MMP2 occurs within the TGN, most likely by furin (Cao, Rehemtulla et al. 2005). However, generated MMP2 appears to be cleaved only once, resulting in secretion of inactive MMP2. Cao et al. hypothesised that this negative regulation of pro-MMP2 activity by furin might provide a paradoxical regulatory mechanism to control MT1-MMP-mediated activation of pro-MMP2 (Cao, Rehemtulla et al. 2005). As around 40% of all MMPs contain a conserved RXKR motif including the membrane type MMPs MT5-MMP and MT2-MMP (Takino, Sato et al. 1995; Pei 1999), it is not surprising that furin activity appears to mediate cleavage of MT3-MMP within the TGN (Kang, Nagase et al. 2002) and shedding of MT5-MMP (Wang and Pei 2001).

Further metalloproteases involved in ECM degradation are synthesised as inactive zymogens and appear to require cleavage through furin or furin-like PCs in order to gain their full activity. These comprise for example the adamalysin metalloproteases ADAM12 and ADAM17, which is also known as tumour necrosis factor α convertase (TACE) (Loechel, Gilpin et al. 1998; Schlöndorff, Becherer et al. 2000), and the secreted ADAMTS-1 (Kuno, Terashima et al. 1999).

5.3. Inhibitors of furin

Due to the implication of furin substrates in many pathological conditions such as Alzheimer's disease, cancer or bacterial and viral infections (see sections 4.4 and 5.2.), the development of specific furin inhibitors would be of potential benefit for treatment of patients. Despite the variety of reported furin inhibitors only a handful of strategies or compounds appear to be furin specific. Hence, most inhibitors like α1-PDX, CMK or peptides based on

poly-basic amino acid motifs (see section 4.6.) act to varying degrees on all furin-like PCs and have to be classified as pan-PC inhibitors.

Prosegment of furin

The prosegment of furin represents the most important endogenous inhibitor of this enzyme. However, as it also inhibits other furin-like PCs to a certain degree, its specificity appears to be limited (see also section 4.6.). Nevertheless, ectopic expression of the prosegment has been used in experimental setups to determine the role of furin in cancer progression. For instance, López de Cicco et al. could show that ectopic expression of furin's prosegment in HNSCC cell lines reduces substrate processing, proliferation and the invasive capability of the cells (Lopez de Cicco, Bassi et al. 2005). Furthermore, introduction of the prosegment of furin in breast cancer and colon cancer cell lines efficiently diminished PDGF-A and IGF1R processing, decreasing cell survival *in vitro* and inhibited tumour growth *in vivo* (Scamuffa, Sfaxi et al. 2014).

Endogenous inhibitors of furin

In addition to the prosegment, other endogenous inhibitors have been reported. The albumin-type serpin proteinase inhibitor 8 was the first naturally occurring furin inhibitor described. It forms a stable complex with soluble furin and direct interaction was shown in platelets by co-immunoprecipitation (Dahlen, Jean et al. 1998; Leblond, Laprise et al. 2006). Another endogenous serpin that blocks furin activity is the inter-α-inhibitor protein, whose application appears to exert a certain protective function against anthrax infection in mice (Opal, Artenstein et al. 2005). Direct interaction of the serpin nexin-1 and furin in endothelial cells blocks ADAM17-mediated shedding of endothelial protein C receptor (Boulaftali, Francois et al. 2013). Furthermore, plasminogen activator inhibitor 1 seems to inhibit intra-Golgi furin activity by forming a stable complex, thus preventing furin-mediated maturation of the insulin receptor and ADAM17 (Bernot, Stalin et al. 2011). Nevertheless, none of the studies commented on the specificity of the proposed furin inhibitors, thus requiring follow up investigations to examine the inhibitory efficacy on other furin-like PCs.

Furin specific nanobodies

In 2012, Zhu et al. proposed the use of furin-specific nanobodies for the therapeutic intervention in pathological conditions that are dependent on furin activity (Declercq, Meulemans et al. 2012). These camelide antibodies were raised against catalytically active furin and appear to inhibit furin but not other PCs in a non-competitive manner. The nanobodies inhibited cleavage of the known furin substrates TGFβ, glypican 3 and diphtheria

toxin, and protected HEK293 cells from cytotoxicity caused by diphtheria toxin. Thus, nanobodies represent the first generation of highly specific inhibitors of furin.

5.4. Furin and rhabdomyosarcoma

The role of furin activity in RMS tumourigenesis and progression has not been investigated in detail, yet. Earlier studies in our laboratory indicated that furin represents the target receptor of the RMS homing peptide RMS-P3 with the minimal binding motif RTRR (Hajdin, D'Alessandro et al. 2010). Furthermore, microarray profiles of PCs in RMS suggested that furin, in contrast to PC1/3, PC2, PACE4 and PC6, has a consistently high expression in RMS cell lines and biopsies. Despite the lack of a direct link between furin activity and RMS, many known furin substrates are overexpressed in RMS and have been associated with RMS progression.

Some of the most prominent examples of furin substrates involved in RMS are components of the IGF signalling pathway. Thus, IGF1R and its ligand IGF1 are not only both processed by furin-like PCs (Duguay, Lai-Zhang et al. 1995; Duguay, Milewski et al. 1997; Khatib, Siegfried et al. 2001), elevated levels of IGF1R have also been correlated with poorer outcome in RMS patients (Blandford, Barr et al. 2006). Similarly, increased levels of the PDGF receptor were associated with a decreased survival rate of patients (Blandford, Barr et al. 2006), indicating a potential importance of furin-mediated PDGF-A and PDGF-B processing (Siegfried, Khatib et al. 2003; Siegfried, Basak et al. 2005). Additionally, TGFβ1 expression appears to be higher in RMS compared to normal skeletal muscle cells and silencing of TGFβ1 resulted in differentiation of RMS cells (Wang, Guo et al. 2010). Hence, cleavage and activation of TGFβ1 through furin (Dubois, Laprise et al. 1995) might favour sustained proliferation of RMS cells. RMS cells were also shown to express various matrix metalloproteases such as MT1-MMP and MMP2, which are implied in dissemination and metastasis (Onisto, Slongo et al. 2005). Furthermore, autocrine VEGF signalling was hypothesised to favour RMS growth (Gee, Tsuchida et al. 2005).

Thus, the broad implication of furin activity in processing and activation of proteins related to RMS progression encouraged us to study in detail the biological role of furin in RMS.

6. Subject of investigation

Rhabdomyosarcoma is the most common paediatric soft tissue sarcoma. Despite advances in risk stratification and therapy options, 5-year overall survival rates are still in need of improvement, particularly for patients with metastatic disease. Furthermore, conventional multimodal treatment strategies often result in acute and long term side effects. Hence, identification and characterisation of novel targets is key to the establishment of complementing treatment options.

Therefore, the main aim of this thesis was to investigate the role of the proprotein convertase furin in RMS maintenance and progression. Since furin proteolytically activates many proteins implicated in RMS-associated signalling pathways, we hypothesised that furin activity is important for proliferation, survival and metastasis of RMS cells.

Firstly, we examined the impact of furin silencing or inhibition on furin substrate processing, migration and invasion in RMS cells *in vitro* and studied RMS tumour growth upon modulation of furin activity *in vivo*.

Secondly, we established a RMS cell model of inducible furin silencing that we further employed to investigate cell viability and apoptosis induction in aRMS cells *in vitro* and tumour growth of Rh30 and Rh4 xenografts *in vivo*. We further analysed furin protein expression in aRMS and eRMS biopsies on a tissue microarray.

Chapter 2

The proprotein convertase furin contributes to rhabdomyosarcoma malignancy by promoting vascularisation, migration and invasion

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This work was primarily done by Patricia Jaaks. Patricia Jaaks contributed the mRNA and protein analysis of paediatric sarcoma cell lines, generation and validation of stable cell lines, migration and invasion assays and protein analysis of furin substrates. Valentina D'Alessandro helped with tumour cell engraftments and analysis of tumour microvessel density and invasion assays. The RD *in vivo* experiment was conducted by Katarina Hajdin. Nicole Grob (Master thesis) contributed furin activity assays and immunoblot analyses. Sina Büel (Master thesis) conducted siRNA based experiments.

Acknowledgements

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1. Introduction

Vast varieties of proteins are synthesized as inactive precursor proteins and require limited proteolysis to be converted into bioactive proteins and peptides. Proproteins are usually cleaved at the general consensus motif R/K-X_n-R/K↓, where n=0, 2, 4 or 6 and X rarely Cys or Pro (Molloy, Bresnahan et al. 1992). Seven mammalian proprotein convertases (PCs) cleaving substrates at dibasic motifs have been described: PC1/3, PC2, furin, PC4, PC5, PACE4 and PC7. In the secretory pathway, PCs mediate the tissue-specific endoproteolytic activation of precursor proteins, including hormones, neuropeptides, growth factors and their receptors, adhesion molecules, bacterial toxins and viral glycoproteins (Seidah and Prat 2012). Consequently, deregulated activity of PCs has been associated with pathological conditions like Alzheimer's disease (Bennett, Denis et al. 2000), endocrinopathies (Jackson, Creemers et al. 1997) or cancer (Khatib, Siegfried et al. 2002; Bassi, Fu et al. 2005). Increased PC activity correlates with higher aggressiveness of diverse cancer types like prostate cancer, colon carcinoma or small cell lung carcinoma (Creemers, Roebroek et al. 1992; Khatib, Siegfried et al. 2001; D'Anjou, Routhier et al. 2011).

Furin was the first described PC and is active within the constitutive secretory pathway (Molloy, Thomas et al. 1994). This calcium-dependent protease cleaves different proteins implicated in cancer progression and metastasis such as the insulin-like growth factor receptor 1 (IGF1R) (Khatib, Siegfried et al. 2001), the growth factors vascular endothelial growth factor C (VEGF-C) and platelet derived growth factor (PDGF) (Siegfried, Basak et al. 2005) and membrane-type 1 matrix metalloprotease 1 (MT1-MMP) (Yana and Weiss 2000; Remacle, Rozanov et al. 2006). Furthermore, elevated levels of furin are found in head and neck cancer, breast, lung and colon cancers (Schalken, Roebroek et al. 1987; Cheng, Watson et al. 1997; Bassi, Mahloogi et al. 2001; Scamuffa, Siegfried et al. 2008).

In a previous study, we identified cell surface furin as a potential receptor for peptides that specifically homed to rhabdomyosarcoma (RMS) cells *in vitro* and *in vivo* (Hajdin, D'Alessandro et al. 2010). RMS represents with around 55% of all cases the most common soft tissue sarcoma in children and adolescents (Kaatsch 2010). Based on histology, paediatric RMS are grouped into two main subtypes: embryonal RMS (eRMS) and alveolar RMS (aRMS). eRMS occurs in around 60-70% of all patients and is associated with a rather good prognosis, whereas 20% of the patients present with aRMS, having a 5-year overall survival of only 20-30% (Van Gaal, Van Der Graaf et al. 2012). Prognosis for patients with metastasis at time of diagnosis is particularly poor with a 5-year overall survival rate of less than 12% (Van Gaal, Van Der Graaf et al. 2012). Complementing or improved treatment strategies are urgently needed and thus many recent efforts have been concentrated on the

identification of key pathways that drive RMS progression. In 80% of aRMS, tumour formation is driven by expression of the chimeric transcription factor PAX3/7-FOXO1 (Sorensen, Lynch et al. 2002; Chen, Stewart et al. 2013; Shern, Chen et al. 2014), which induces expression of a specific gene expression signature (Wachtel, Dettling et al. 2004; Davicioni, Finckenstein et al. 2006; Williamson, Missiaglia et al. 2010). Many receptor tyrosine kinases are direct targets of PAX3/7-FOXO1, including IGF1R, VEGFR and PDGFR (Epstein, Song et al. 1998; Cao, Yu et al. 2010), and RMS progression is characterised by aberrant activation of growth factor signalling pathways (Crose and Linardic 2011). As furin is involved in the maturation and activation of many components of these pathways, we hypothesized that furin activity is important for maintenance of the malignant phenotype of RMS cells. Therefore, we assessed the expression levels of furin and other PCs in paediatric sarcoma cell lines and generated RMS cell lines with modulated furin activity. We subsequently used these cell lines to examine tumour growth *in vivo* and to assess processing of furin substrates, migration, and invasion *in vitro*. Our investigations showed that furin promotes tumour growth as well as migration and invasion abilities in RMS by cleaving key proteins involved in RMS cell growth, tumour vascularisation, and cell motility and invasiveness. Therefore, we propose the PC furin as new therapeutic target for treatment of RMS.

2. Material and methods

Ethics Statement

All animal experiments were approved and monitored by the veterinary office of the Canton of Zurich according to the Swiss Federal Law.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Data are expressed as mean ± standard deviation (SD). Statistical significance was tested with unpaired two-tailed Student's t-tests, or for multiple comparison analysis of variance (ANOVA). The differences were considered to be significant if $p<0.05$.

Cell lines and cell culture

Osteosarcoma cell lines SAOS, LM5, HOS, 143B, MG63, M8, HU09 and M132 were kindly provided by Roman Muff (University Hospital Balgrist, Zurich, Switzerland). Ewing sarcoma cell lines TC71, A673, RD-ES, SK-ES and SKNMC, as well as the RMS cell line RUCH-2, were kindly provided by Beat Schäfer (University Children's Hospital Zurich, Zurich, Switzerland). Other RMS cell lines were kindly provided by following sources: Rh3 and Rh5 by Susan Ragsdale (St. Jude Children's Research Hospital, Memphis, TN, USA); RD, Rh36, Rh18, Rh4 and Rh41 by Peter Houghton (The Research Institute at Nationwide Children's Hospital, Columbus, OH, USA); TTC442 and Birch by Timothy Triche (Children's Hospital Los Angeles, Los Angeles, CA, USA); NRS-1 (RIKEN Cell Bank); RMS and SCMS-RMZ by Janet Shipley (The Institute of Cancer Research, London, UK); Rh28 by Corinne Linardic (Duke University School of Medicine, Durham, NC, USA); RMS13 by Roland Kappler (LMU, Munich, Germany); CW9019 by Soledad Gallego (Hospital Universitari Vall d'Hebron, Barcelona, Spain); KFR by Jindrich Cinatl (Frankfurter Stiftung für krebskranke Kinder, Frankfurt, Germany); RC2 by Pier-Luigi Lollini (University of Bologna, Bologna, Italy) and RhJT by Scott Diede (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Rh30 cells were purchased from ATCC (LGC Promochem, Molsheim Cedex, France). All cells were maintained under proliferating conditions in high glucose DMEM medium (Sigma-Aldrich, Buchs, Switzerland) supplemented with 10% fetal calf serum (Bioconcept, Switzerland) in 5% CO_2 at 37 °C.

Plasmids and Transfections

pcDNA3.1(+) vectors encoding the full length furin (fur) (Bassi, Mahloogi et al. 2003) or α1-AT Portland (pdx), were generous gifts of Andres JP Klein-Szanto (Fox Chase Cancer

Center, Philadelphia, PA). For stable transfections, 2.5-5x10^5 RD, Rh4 or Rh30 cells were transfected with 2 µg DNA according to the jetPRIME™ short protocol (Polyplus Transfection, Illkirch, France). Selection with 1 mg/ml G418 (Promega, Wallisellen, Switzerland) was started 48h post transfection and was continued for 14 days. RMS cells transfected with empty pcDNA3.1(+) vector were used as control in qRT-PCR experiments.

Transient and stable silencing of furin

To transiently silence furin, 10 nM siRNA (Silencer Select, Ambion, Austin, Texas) were reverse transfected into RD cells using the transfection reagent INTERFERin (Polyplus Transfection, Illkirch, France) according to manufacturer's instructions. Following siRNAs were used: scrambled (scr, ID: AM4637), siFurin1 and siFurin2 (siFur1, ID: s9987; siFur2, ID: s9988). For stable silencing of furin in RD, Rh4 and Rh30 cells, mission shRNA lentiviral particles (Sigma-Aldrich) based on a pLKO.1 plasmid backbone were used. Lentiviral particles of five clones (SHCLNV-NM 002569 Human, TRCN0000075238-42) targeting different sequences of furin RNA or lentiviral particles non-target shRNA (SHC002V) were added at an MOI of 5 to RMS cells for 24h. Selection was started on day 5 with 2 µg/ml of puromycin (Invitrogen, Basel, Switzerland). Non-target shRNA RMS cells were used as control in qRT-PCR experiments.

Animal models

To study *in vivo* growth, 5x10^6 RMS cells in 150 µl of PBS were injected s.c. into NOD/Scid IL2rg$^{-/-}$ mice (Charles River) at 5-6 weeks of age, under anaesthesia induced by intraperitoneal injection of 100 mg/kg ketamine (Ketalar, Parke-Davis, Morris Plains, NJ) and 16 mg/kg xylazine (Rompun, Bayer HealthCare, Leverkusen, Germany). For measurement of the tumour size, both diameters (d) of the spherical tumours were measured with a caliper and the tumour volume was calculated using the formula $V = (4/3)\cdot\pi\cdot r^3$, whereby $r = ((d_1+d_2)/4)$. Mice were sacrificed when a tumour size of 1000 mm^3 was reached. Mice were perfused with PBS after terminal anaesthesia with 420 mg/kg pentobarbital (Esconarkon; Streuli Pharma, Uznach, Switzerland). Tumours were dissected, fixed for 24 hours in 4% PFA (Thermo Scientific, Switzerland) and embedded in paraffin, or stabilised in 30% sucrose (Sigma-Aldrich, Basel, Switzerland) and frozen in O.C.T embedding medium (Leica Microsystems, Heerbrugg, Switzerland), as indicated.

Immunofluorescence

Immunofluorescence to detect angiogenic blood vessels in RMS xenograft tumours was performed on 5 µm fresh frozen section from O.C.T. embedded samples. Sections were washed in TBS/0.2% Tween-20 for 15 min and stained with CD31 antibody (550274, BD Pharmingen, Allschwil, Switzerland) diluted 1:100 in antibody diluent solution (Zytomed Systems GmbH, LabForce, Nunningen, Switzerland). Secondary Alexa Fluor 594-labelled goat anti-rat IgG was used for detection (A-11007, Invitrogen). Sections were washed twice with PBS and mounted with Vectashield Mounting medium containing DAPI (Reactolab SA, Servion, Switzerland).

Immunohistochemistry

Immunohistochemistry of PFA-fixed tumours was performed by Sophistolab (Muttenz, Switzerland) on an automated Leica BondMax system using Bond Polymer Refine Detection (DS9800, Leica Microsystems Newcastle, UK) including all buffer-solutions from Leica, processed according to the manufacturer's instructions. Paraffin slides were dewaxed, pretreated with ER-Solution 2, and incubated with the following antibodies: polyclonal rabbit anti-furin (ab28547, Abcam, Cambridge, UK) used at a dilution of 1:3000 after pretreatment for 10 min at 95 °C; anti-CD31 (ab28364, Abcam) used at a dilution of 1:100 after antigen pretreatment for 20 min at 100 °C.

Quantitative RT-PCR

Total RNA was extracted from RMS cells or tumour tissue using the RNeasy Kit (Qiagen, Hombrechtikon, Switzerland) including a DNase treatment step. 1 µg total RNA was reverse-transcribed with random primers using the Omniscript Reverse Transcription Kit (Qiagen). qRT-PCR detection of furin, α_1-PDX and the house keeping gene GAPDH was performed with assay-on-demand Hs00965485_g1, Hs01097800_m1 or Hs99999905_m1, respectively (Applied Biosystems, Basel, Switzerland) and normalized to GAPDH.

Immunoblot

RMS cells were lysed in denaturing buffer (50 mM TrisHCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$, 10 mM $C_3H_7Na_2O_6P$, 1 mM sodium orthovanadate) supplemented with 1 mM PMSF (phenylmethylsulfonyl fluoride) and Roche Complete Protease inhibitor (Roche, Rotkreuz, Switzerland). Cell lysates were centrifuged for 10 min at 10'000 rpm, the supernatant was collected and protein concentrations were

determined in a Bradford assay (Bio-Rad laboratories, Reinach, Switzerland). Total cell extracts were separated on 4-12% NuPAGE Bis-Tris gels (Invitrogen) and blotted on nitrocellulose membranes (0.1 µm; Schleicher & Schuell, Dassel, Germany). Blots were blocked with 5% milk in TBS/0.05% Tween-20, incubated with the first antibody overnight at 4 °C, and with the corresponding HRP-conjugated secondary antibody for 1h at RT. Enhanced chemiluminescence detection system SuperSignal West Femto (Pierce, Perbio Science, Lausanne, Switzerland) was used for signal detection. The following antibodies were used: anti-furin mouse monoclonal MON-152 (ALX-803-017-R100, 1:750, Alexis Corporation, Lausen, Switzerland), anti-VEGF-C rabbit polyclonal (sc-25783, 1:200; Santa Cruz, LabForce, Switzerland), anti-IGF1Rβ rabbit polyclonal (9750S, 1:1000, Cell Signaling, Bioconcept), anti-MT1-MMP rabbit polyclonal (NG1726963, 1:750, Millipore), anti-PDGF goat polyclonal (06-127, 1:500, Millipore), anti-pAkt polyclonal rabbit (9271S, 1:1000, Cell Signaling, Bioconcept) and anti-Akt polyclonal rabbit (9272, 1:1000, Cell Signaling, Bioconcept). Mouse anti-β-actin (A5316, 1:5000, Sigma-Aldrich) or anti-α-tubulin (T4026, 1:1000, Sigma-Aldrich) were used for loading controls.

Furin activity test

To measure the activity of furin, an immobilised furin assay was performed as described previously (Bourne and Grainger 2011). 10^6 RMS cells were treated with 5x lysis/ reaction buffer (500 mM HEPES pH 7.0, 2.5% Triton X-100, 5 mM calcium chloride, 5 mM β-mercaptoethanol), incubated on ice for 10 min and centrifuged. Black FluoroNunc 96 well plates (MaxiSorp surface, Nunc, Thermo Scientific) were coated with goat anti human furin antibody (AF1503, R&D Systems) at 10 µg/ml in 50 mM Na_2CO_3, pH 9.6 (50 µl/ well) for 8h at RT, protected from light. After blocking for 1h at RT (0.5% sucrose, 0.5% Tween-20 in PBS), cell lysates were plated in triplicates and incubated over night at 4 °C. Wells were washed, 180 µL 1.1x lysis buffer per well added, and the reaction was started by adding 20 µl/well of 1 mM furin fluorogenic Boc-RVRR-AMC (ALX-260-040 Alexis, Enzo Life Sciences). Fluorescence (ex: 380 nm; em: 460 nm) was measured using a Bio-TEK Multi-Mode Microplate Reader (Witec AG, Luzern, Switzerland).

Cell migration

RMS cells were cultured to 90% confluency in 6-well plates and three separate wounds per well were introduced with a 200 µl pipette tip. Six pictures per well were taken at 10x magnification at different time points (0, 4, 16, 24 hours), analysed with the program TScratch

(Geback, Schulz et al. 2009) and the percentage of closed wound was calculated over the time point 0h.

Invadopodia assay

Invadopodia assays were performed following the manufacturer's instructions (ECM670, Millipore, Zug, Switzerland). For the experiment, $1.2x10^3$ cells were seeded onto FITC-labeled gelatin into 8-chamber slides and incubated at 37 °C for 24h or 48h. Cells were fixed with 4% formaldehyde for 30 min, washed and stained with a solution of TRITC-phalloidin (2 µg/ml) and DAPI (1 µg/ml) in fluorescent staining buffer (PBS with 2% blocking serum and 0.25% Triton X-100) for 1h at RT, protected from light. Coverslips were mounted on the slides using hard set mounting medium (Reactolab) and pictures were taken at 20x magnification with a fluorescence microscope (Axioskop 2 Mot, Zeiss, Feldbach, Switzerland). Image analysis was performed using the program ImageJ (Schneider, Rasband et al. 2012).

IGF1 stimulation

RD cells were transiently transfected with anti-furin siRNA (siFur1 and siFur2) or control (scr) siRNA, as described above. After 24h of starvation (DMEM, 0.2% FBS), cells were stimulated for 10 min with 50 ng/mL IGF1 (ab73455, Abcam). Cells were washed with ice-cold PBS and snap frozen in liquid nitrogen. Whole cell lysates were prepared as described above and IGF1Rβ, Akt and phosphorylated Akt were analysed by immunoblotting.

3. Results

Furin is expressed and active in RMS cell lines

To assess the presence of furin and other PCs in paediatric sarcomas we examined the mRNA levels of all nine known PCs in 5 Ewing sarcoma, 8 osteosarcoma and 20 rhabdomyosarcoma (RMS) cell lines. Overall, RMS cell lines showed higher levels of PCs than Ewing sarcoma or osteosarcoma cell lines. Particularly furin, PACE4 and S1P were highly expressed, with furin being the PC with the most consistently elevated mRNA level (Figure 1A and S1).

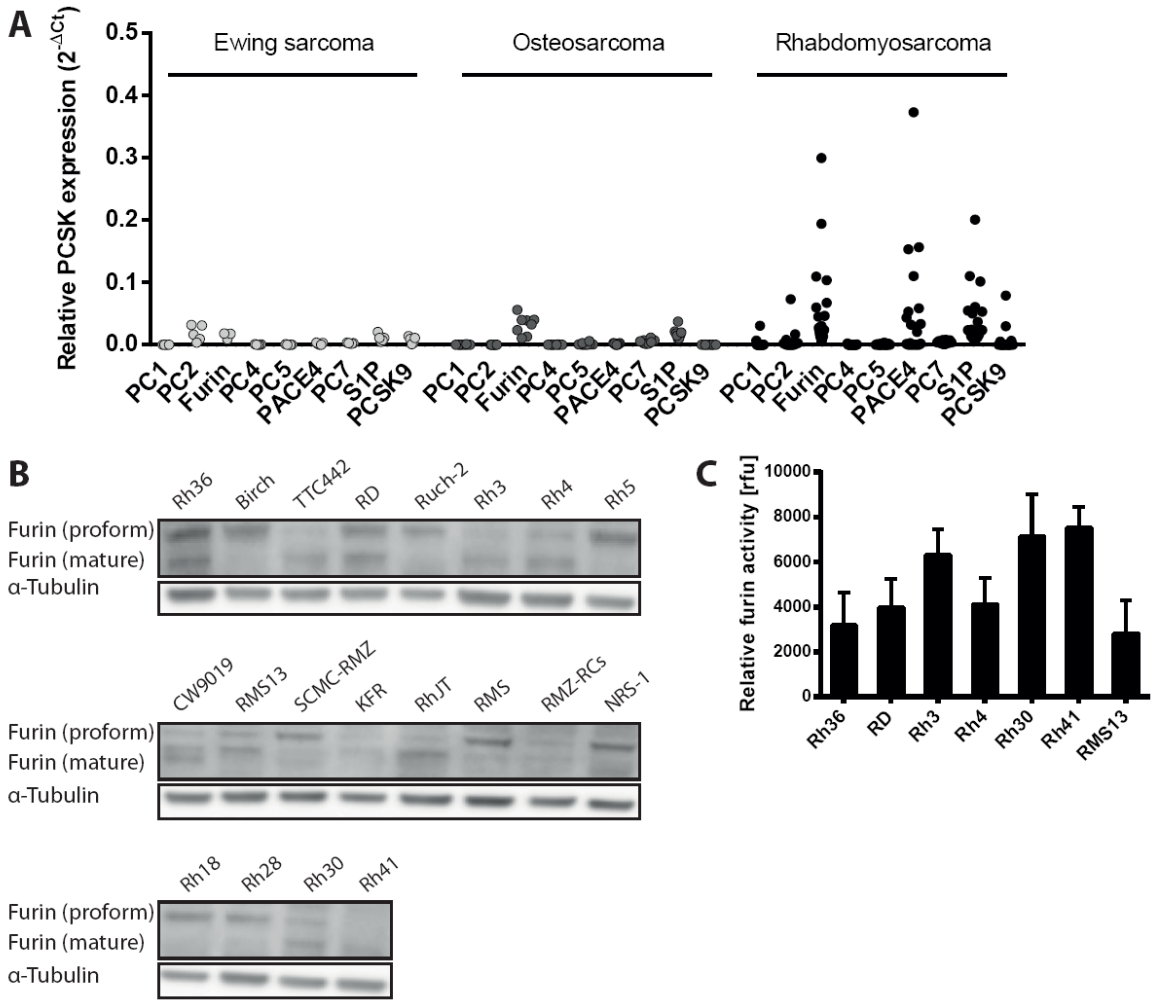

Figure 1. Furin is highly expressed in rhabdomyosarcoma (RMS) cell lines. A) mRNA levels of all nine proprotein convertases (PCs) were determined by qRT-PCR in 5 Ewing sarcoma, 8 osteosarcoma and 20 rhabdomyosarcoma (RMS) cell lines. Shown are levels relative to GAPDH expression. B) Protein levels of proform and mature furin were assessed by immunoblotting in 20 different RMS cell lines. C) Endogenous furin activity of selected RMS cell lines: Rh36, RD (eRMS), Rh3, Rh4, Rh30, Rh41 and RMS13 (aRMS). Furin was captured from cell lysates on anti-furin antibody coated plates and furin activity was measured by addition of the fluorogenic substrate Boc-RVRR-AMC after 6h. Displayed are values normalised to background control.

Subsequently, we analysed maturation status of furin protein in all RMS cell lines and found detectable amounts of mature furin in approximately half of the cell lines tested (Figure 1B). From those, we chose seven cell lines (two eRMS, five aRMS) and measured activity of furin based on a fluorogenic substrate assay. These experiments showed that levels of mature furin protein detected by immunoblot correlate with furin activity *in vitro*, thus indicating that immunoblots can be used to predict furin protease activity *in vitro* (Figure 1C).

Establishment and validation of RMS cells with modulated furin activity

To investigate the function of furin in RMS, we selected two cell lines, one for each RMS subtype, Rh30 (aRMS) and RD (eRMS), and generated stable cell lines with either increased furin activity by overexpression of furin (fur), or with decreased furin activity by overexpression of the pan-PC inhibitor α1-antitrypsin Portland variant (pdx). In addition, to achieve a specific inhibition of furin, we downregulated furin expression with two distinct shRNAs targeting furin (shFA and shFE). Furin and pdx overexpression, as well as furin silencing by shRNA, were first validated at the mRNA level by qRT-PCR. As expected, RMS cells overexpressing furin (RMS fur) showed an increased level of furin expression compared to the respective empty vector cells (Figure 2A; Rh30 fur: 5.5-fold, RD fur: 8-fold). *De novo* expression of pdx was confirmed by qRT-PCR (data not shown). Both furin shRNAs, shFA and shFE, on the other hand decreased furin expression around 5-fold over control (scr) shRNA (Figure 2A).

Next, furin protein levels were analysed by immunoblotting, confirming overexpression of furin in Rh30 fur and RD fur, wild type levels in Rh30 pdx and RD pdx, and decreased protein levels for both silenced Rh30 and RD (Figure 2B). In order to verify the correlation between the presence of furin protein and its activity, we performed a whole cell lysate specific furin activity assay (Bourne and Grainger 2011). Furin overexpression led to a significant increase in furin activity (Figure 2C; Rh30 fur: 1.8-fold, RD fur: 2.5-fold). Inhibition of furin through pdx expression, or reduction of furin expression by shRNA both efficiently reduced furin activity (Figure 2C). In order to exclude possible compensation of lowered furin activity through increased expression of related PCs, we analysed mRNA levels of all PC family members and did not observe any compensatory increase of other PCs upon loss of functional furin (data not shown). Thus, successful modulation of furin activity in the aRMS and eRMS cell lines Rh30 and RD could be confirmed at mRNA, protein and activity levels, allowing the use of these cell lines for further investigations on the role of furin in RMS progression.

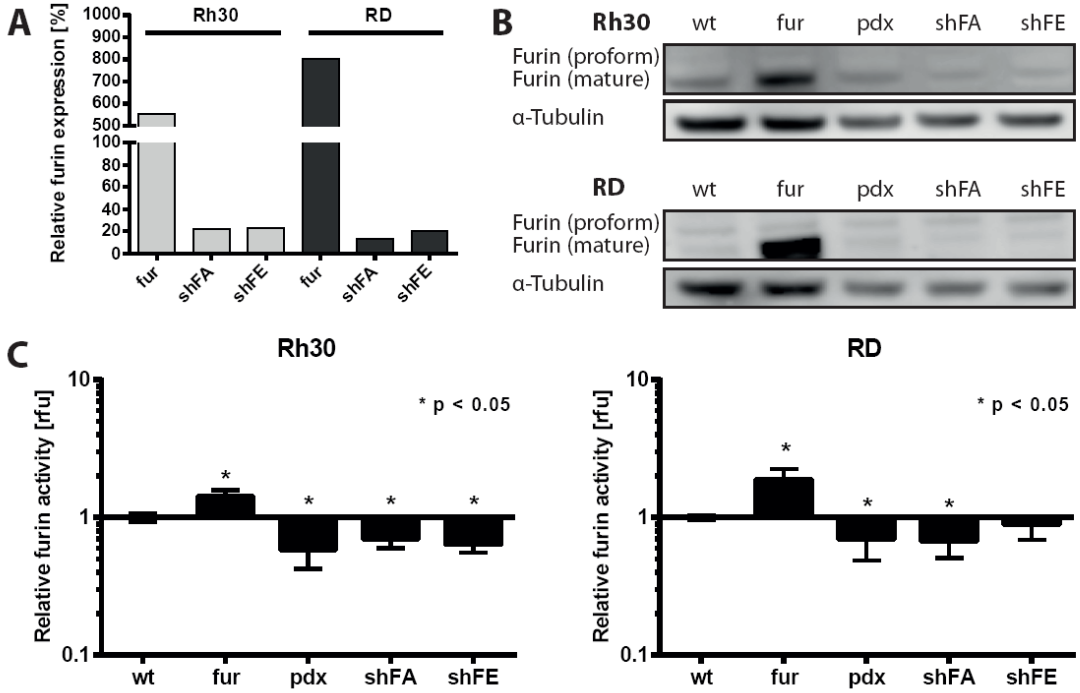

Figure 2. Generation and validation of RMS cells with stable modulation of furin. Rh30 and RD cells were stably transfected with pcDNA3.1(+) full length furin (fur) or α1-AT Portland (pdx). shRNA lentiviral particles were employed for stable furin silencing (shFA, shFE). A) Furin mRNA levels were quantified by qRT-PCR. Expression levels were normalised to GAPDH and to furin expression of the respective control cells (empty vector or scrambled shRNA). B) Levels of furin protein in stable Rh30 and RD cell lines were assessed by immunoblotting with antibody MON-152. C) Assessment of furin activity in furin modulated cell lines. The results were normalised over the respective wt cells on a $\log_{10}$ scale. Data represent three independent experiments. Depicted are mean values ± SD. Two-way ANOVA, $p<0.05$.

Furin activity correlates with tumour growth *in vivo*

Having successfully established Rh30 and RD cells with different levels of furin activity, we used these cell lines to investigate the impact of furin on RMS tumour growth *in vivo*. RMS tumours were generated from the different RMS cell lines by subcutaneous engraftment in NOD/Scid IL2rg$^{-/-}$ mice, and tumour growth was monitored. Wild type aRMS Rh30 cells grew faster than wild type eRMS RD cells; Rh30 tumours were followed over a period of 40 days, while RD tumours were followed over a period of 20 weeks. No difference in tumour growth was observed between Rh30 wt and Rh30 fur, whereas decreased furin activity resulted in a clear delay in tumour growth (Figure 3A); Rh30 pdx, Rh30 shFA and Rh30 shFE tumours took approximately 1.5-times longer to reach a size of 500 mm^3 compared to Rh30 wt (Figure 3A). The time period to reach 500 mm^3 was 22 days for Rh30 wt and Rh30 fur, 30 days for Rh30 pdx, and 35 days for Rh30 shFA and Rh30 shFE. The same trend was observed for RD tumours, whereby RD tumours with decreased furin activity grew slower than RD wt tumours and took almost 30% longer to reach a size of 500 mm^3 (Figure 3B; RD wt: 14 weeks, RD pdx:

20 weeks). Remarkably, an increase in furin expression led to faster tumour growth in RD xenografts; RD fur tumours reached a size of 500 mm^3 in 8 weeks compared to 14 weeks required by RD wt to reach the same size, which corresponds to a 40% decrease (Figure 3B). This acceleration in tumour growth upon furin overexpression was not observed in the more aggressive Rh30 tumour model. These results suggest that furin activity supports a rapid tumour growth in RMS xenografts. However, in an already very fast growing model, such as Rh30, tumour growth cannot be further increased by furin overexpression.

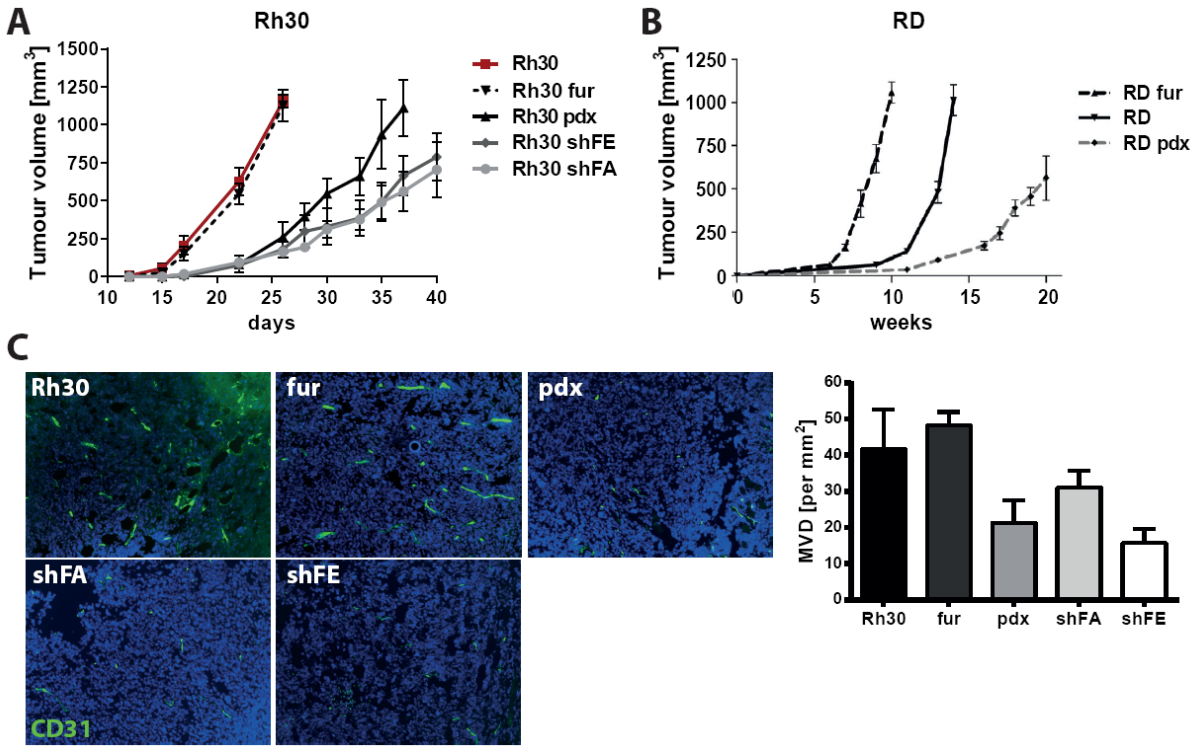

Figure 3. Furin activity positively correlates with tumour growth and microvessel density. 5 million RMS cells with stable modulation of furin activity were injected s.c. into NOD/Scid IL2reg$^{-/-}$ and tumour growth was monitored over time. A) Tumour growth in aRMS Rh30 xenografts. Tumours took on average 20-35 days to reach a size of 500 mm^3. Each group consisted of 6 mice. B) Tumour growth in eRMS RD xenografts. Tumours took on average 7-19 weeks to reach a size of 500 mm^3. Each group consisted of 10 mice. C) Histological analysis of microvessel density in Rh30 xenografts. Tumours were collected when they reached a size of 200-400 mm^3 and sections were stained for CD31. Microvessel density was assessed as vessels per mm^2. Right: Representative pictures are shown (magnification: 10x). Left: The graph shows the quantification averaged per picture. Depicted are mean values ± SD.

Assessment of furin protein levels by immunoblotting post tumour extraction revealed that overexpression of furin in Rh30 fur *in vivo* could only moderately increase furin levels as compared to tumours derived from Rh30 wt cell lines, in contrary to what was observed *in vitro* (Figure 2B and S2). This might be due to an increase of furin protein levels in Rh30 tumours grown *in vivo*. Importantly, immunoblots showed that the silencing of furin in shFA and shFE

was maintained *in vivo* in Rh30 cells derived tumours (Figure S2). Taken together, these results support the hypothesis that furin activity contributes to RMS growth *in vivo*. Moreover, furin silencing and inhibition led to delayed tumour onset rather than to a complete tumour growth inhibition, hinting at a role of furin in early steps of tumour growth *in vivo*.

Furin affects microvessel formation in Rh30 xenografts *in vivo*

Since we observed a delayed initiation of tumour growth upon reduction of furin activity, we were interested to elucidate the involved molecular mechanisms. Therefore, we analysed the microvessel density (MVD) on sections from Rh30 tumours by immunostaining for CD31. Furin overexpression in Rh30 fur resulted in a slight increase in MVD compared to wild type Rh30 (Figure 3C; Rh30 wt: 40 vessels/mm^2, Rh30 fur: 50 vessels/mm^2). Conversely, reduced furin activity in Rh30 cells led to lower MVD (Figure 3C; Rh30 pdx: 25 vessels/mm^2, Rh30 shFA: 30 vessels/mm^2, and Rh30 shFE: and 20 vessels/mm^2). These results suggest a possible role of furin in the recruitment of new blood vessels at the early stage of RMS tumour growth, which, in turn, might account for the differences in tumour growth onset that were observed.

Furin activity favours migration and invasion *in vitro*

In order to further investigate the contribution of furin to the malignant phenotype of RMS cells, we studied migration and invasion abilities of Rh30 and RD cells with distinct furin activities *in vitro*. To investigate cell migration *in vitro*, we assessed wound closure over a timeframe of 24h. Rh30 wt cells migrated in general a little faster than RD wt cells (Figure 4A and B). In both cell lines, overexpression of furin led to an accelerated wound closure compared to the corresponding wild type cells. This effect was more pronounced in RD fur as compared to Rh30 fur. In contrast, inhibition of furin activity through expression of pdx or decreased expression by silencing of furin resulted in delayed wound closure for both Rh30 and RD cells. These results indicate a positive correlation between furin activity and the ability of RMS cells to migrate.

Next, we monitored the effect of furin on the ability of Rh30 and RD cells to invade a matrix. This was assessed with an invadopodia assay that allows assessment of cellular invasiveness by measuring the degradation of fluorescent gelatin. In addition to the previously described stable cell lines with modulated furin, we also used the potent PC inhibitor decanoyl-RVKR-chloromethylketone (CMK) in this assay. In both RMS cell lines, Rh30 and RD, reduction of furin activity, either in stably modified cells (pdx or shRNA) or in wild type cells treated with CMK, resulted in diminished matrix degradation (Figure 4C and D). The gain in furin activity, on the other hand, led to increased degradation of the matrix in RD fur cells

compared to RD wt. Surprisingly, Rh30 fur were less able to degrade gelatin than Rh30 wt, although not in a statistically relevant manner. This result is in line with previous *in vitro* and *in vivo* observations, where we did not observe an impact of furin overexpression in Rh30 on the cell phenotype, suggesting that in Rh30 cells furin activity appears to be already at a plateau and cannot be further increased. Taken together, these results point at a role of furin activity in migration and invasion processes in RMS cells.

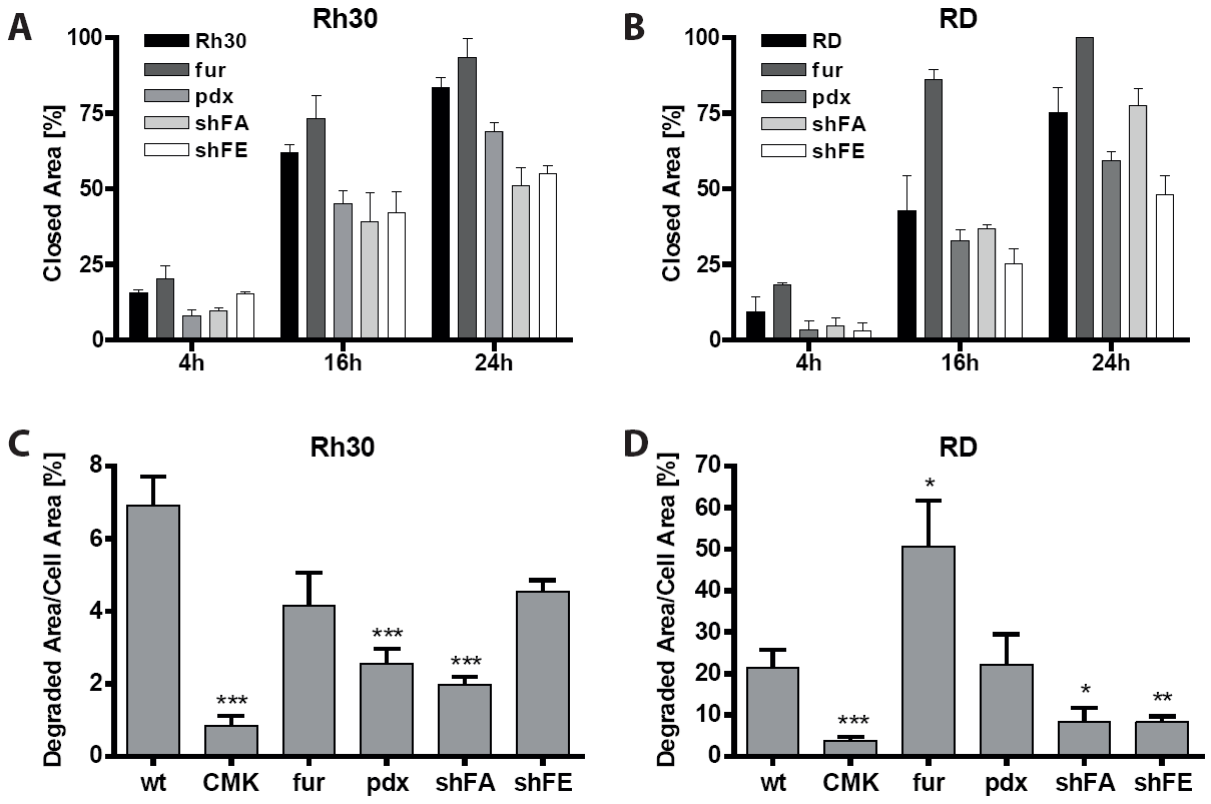

Figure 4 Migration and invasion of RMS cells is influenced by furin activity. A) Impact of furin activity on wound healing. Rh30 and RD cells with stable modulation of furin activity were used in a scratch assay. Pictures were taken at 0h, 4h, 16h and 24h, and wound healing was assessed over time 0h using the program TScratch. Depicted are mean values ± SD. B) Effect of furin activity on RMS cell invasiveness. Rh30 and RD cells (wt, fur, pdx, shFA and shFE) were cultured on fluorescently labeled gelatin-coated 8-chamber slides for 24h and 48h, respectively. Treatment with 50 µM of the pan-PC inhibitor decanoyl-RVKR-chloromethylketone (CMK) was started 16h prior plating of the cells. Immunofluorescent pictures were taken and percentage of invasion was calculated based on degradation of fluorescent gelatin using the programme ImageJ. Depicted are mean values ± SD. Student t-test, $p<0.05$ *, $p<0.01$ **, $p<0.005$ ***.

Furin regulates maturation of substrates important in RMS progression

To decipher the furin substrates involved in tumour growth, migration and invasion in RMS cells, we examined by immunoblotting the maturation status of IGF1Rβ, VEGF-C, PDGF-BB and MT1-MMP in Rh30 wt and RD wt cells upon inhibition of furin activity by the PC inhibitor CMK (Figure 5A). In the case of IGF1Rβ and PDGF-BB, we observed an

accumulation of precursor and decrease in mature proteins upon inhibition of furin with CMK. For VEGF-C and MT1-MMP reduced furin activity resulted in lower levels of mature and precursor protein, suggesting enhanced degradation of unprocessed protein proforms. Thus, in RMS cells PCs seem to be involved in the maturation of all four investigated furin substrates. In addition to the use of the inhibitor CMK, we examined the maturation of IGF1Rβ in Rh30 and RD cells with stable modulation of furin activity by immunoblotting (Figure 5B). In both cell lines, we observed a slight increase in cleaved IGF1Rβ upon overexpression of furin. Conversely, a decrease of furin activity, either by expression of the inhibitor pdx or by silencing of furin, led to an accumulation of IGF1Rβ precursor and lower levels of mature IGF1Rβ (Figure 5B).

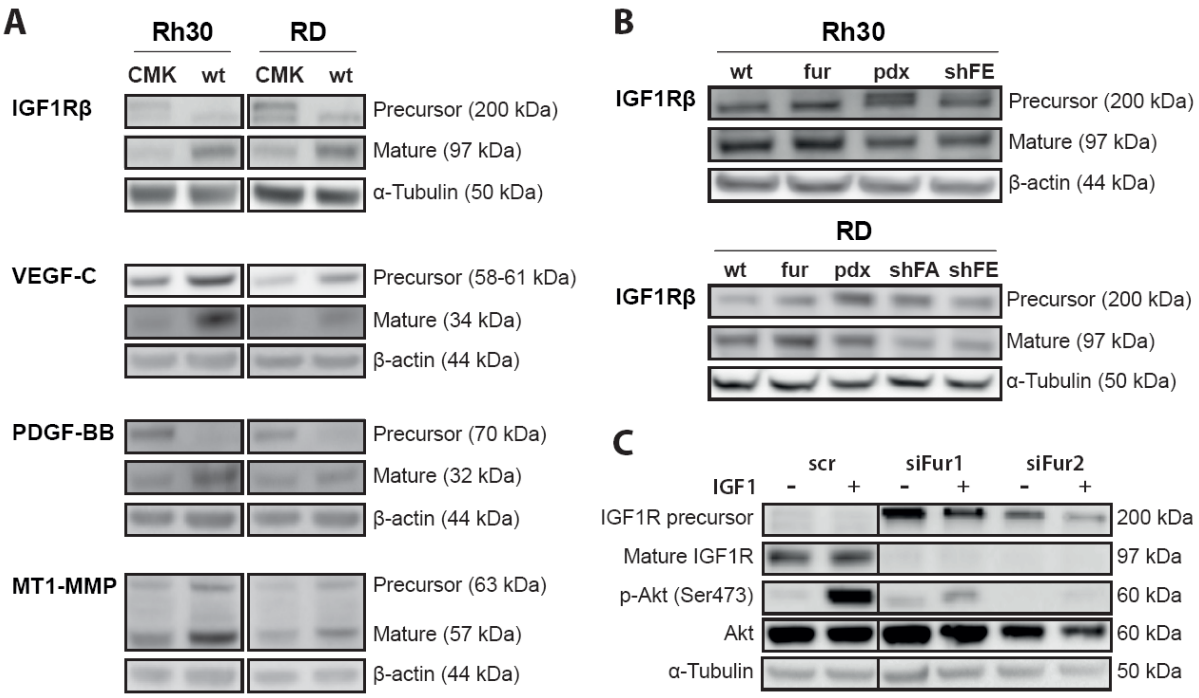

Figure 5 Maturation of furin substrates and IGF1R signalling are furin activity dependent in RMS cells. A) Maturation of furin substrates upon treatment with pan-PC inhibitor CMK. Rh30 and RD cells were treated with 50 μM CMK for 16h and maturation status of the furin substrates IGF1Rβ, VEGF-C, PDGF-BB and MT1-MMP were examined by immunoblotting. B) IGF1Rβ maturation in Rh30 and RD cell lines. Levels of precursor and mature IGF1Rβ were analysed in stable Rh30 and RD cell lines (wt, fur, pdx, shFA and shFE) by immunoblotting. C) IGF signalling in RD cells upon transient furin silencing. RD cells were transiently transfected with control (scr) or anti-furin siRNA (siFur1 and siFur2) and serum starvation was started 24h post transfection. Cells were stimulated for 10 min with 50 ng/mL IGF1 48h post transfection and maturation status of IGF1Rβ and phosphorylation of Akt (Ser473) were assessed by immunoblotting.

In order to examine the impact of furin activity on IGF signalling in RMS cells, and to exclude any type of effect arising during cell selection required for shRNA, we transfected RD cells with specific siRNA against furin (siFur1 and siFur2) and stimulated the cells with

50 ng/mL IGF1, 48h post transfection. As expected, silencing of furin severely impaired maturation of IGF1Rβ, leading to a decrease in mature form and accumulation of IGF1Rβ proform. Control siRNA transfected RD cells showed a high level of Akt phosphorylation upon stimulation with IGF1. In contrast, silencing of furin and subsequent loss of IGF1Rβ maturation mostly prevented stimulation of Akt phosphorylation, indicating an inability of RMS cells to trigger IGF signalling in the absence of furin activity (Figure 5C). Phosphorylation of Erk1/2 was generally not stimulated in RD cells upon treatment with IGF1 (data not shown). Taken together our data suggest that furin activity is necessary to ensure processing of IGF1Rβ, VEGF-C, PDGF-BB and MT1-MMP and that maturation of IGF1Rβ by furin is required to maintain IGF signalling. Thus, furin has an important role in promoting the malignant phenotype of RMS cells by supporting the maturation and activation of proteins involved in, or enabling, cellular abilities such as proliferation or matrix degradation.

4. Discussion

In this study, we show that furin activity plays a crucial role in RMS progression *in vitro* and *in vivo*. Loss of furin activity, through RNA interference or application of pan-PC inhibitors, delayed tumour growth *in vivo* and decreased migration and invasion *in vitro*. Furthermore, reduced furin activity resulted in diminished processing of important furin substrates and abrogation of IGF signalling.

Upon engraftment of mice with Rh30 and RD cells having stably silenced furin expression or with expression of the pan-PC inhibitor α1-PDX/pdx (Anderson, Thomas et al. 1993) we observed delayed RMS tumour growth and we hypothesise this could be the result of reduced growth factor signalling and/or impaired vessel formation. We could show that proteolytic activity of furin is required to process the potent mitogen PDGF-BB and the growth factor receptor IGF1R in RMS cells, the latter resulting in decreased IGF signalling upon stimulation with IGF1. Similarly, furin inhibition in carcinoma cells diminishes PDGF-A and IGF1R processing and abolishes stimulation of related signalling pathway (Scamuffa, Sfaxi et al. 2014). Our findings suggest that furin plays a role in RMS cell proliferation and thus reduced levels of furin likely impair tumour growth *in vivo*. In order to grow beyond a certain size, a tumour has to initiate angiogenesis, a step in tumourigenesis that is known as the angiogenic switch (Bergers and Benjamin 2003). Furin activity has been shown to promote vascularisation of tumours (Khatib, Siegfried et al. 2001; Lopez de Cicco, Watson et al. 2004; Lopez de Cicco, Bassi et al. 2005), whereby stimulation of vessel formation is dependent on secretion of different angiogenic growth factors like VEGF-C and VEGF-D, both of which require processing through furin or furin-like PCs to stimulate VEGF signalling and angiogenesis or lymphangiogenesis (Joukov, Sorsa et al. 1997; McColl, Paavonen et al. 2007). Accordingly, maturation of VEGF-C was shown to be necessary for subsequent tumour formation (Siegfried, Basak et al. 2003). Likewise, secretion of mature PDGF-BB favours tumour angiogenesis through paracrine stimulation of PDGFR-β on endothelial cells, leading to proliferation, migration and sprouting (Xue, Lim et al. 2012). Hence, decreased VEGF-C and PDGF-BB processing, as confirmed *in vitro*, is likely responsible for reduced microvessel density *in vivo*, and delayed the angiogenic switch. We therefore suggest that the shift in initial tumour growth observed in RMS tumours with reduced furin activity can be attributed to impaired furin substrate processing, which reduced growth factor signalling and angiogenesis.

Furthermore, we found that presence of furin is necessary to maintain the migratory and invasive behaviour of RMS cells *in vitro*. Hence, we observed reduced ability of wound healing and gelatin degradation, which correlated with decreased processing of the furin substrate MT1-MMP. Furin is also responsible for the activation of other matrix

metalloproteases including ADAMs (Loechel, Gilpin et al. 1998) and ADAM-TS (Kuno, Terashima et al. 1999) and indirectly mediates MMP2/gelatinase A activation, since active MT1-MMP is the main enzyme responsible for processing of pro-MMP2 on the cell surface (Ohuchi, Imai et al. 1997). Additionally, autocrine PDGF signalling was shown to promote the metastatic potential of breast cancer and pancreatic cancer cells (Jechlinger, Sommer et al. 2006; Weissmueller, Manchado et al. 2014). Therefore, furin-mediated PDGF processing in RMS cells might further favour their metastatic behaviour. Thus, several lines of evidence support the notion that PCs, and in particular furin, activate metastasis-associated proteins. For instance, furin-mediated catalytic activation of MT1-MMP has been linked to progression of head and neck squamous cell carcinomas (Bassi, Lopez De Cicco et al. 2001; Bassi, Mahloogi et al. 2001). Similar to our studies, others showed that inhibition of furin decreases cell motility, migration and invasiveness of breast cancer, lung adenocarcinoma, fibrosarcoma and osteosarcoma cells *in vitro* (Lapierre, Siegfried et al. 2007; Coppola, Bhojani et al. 2008; Liu, Li et al. 2014; Ma, Fan et al. 2014) and proposed furin inhibition for prevention of metastasis (Scamuffa, Siegfried et al. 2008).

In conclusion, our study identifies a role of furin activity in RMS progression and metastasis, which is mediated via proteolytic activation of key substrates involved in proliferation, motility and dissemination of RMS cells. We confirmed furin-dependent processing of IGF1R, PDGF-BB, VEGF-C and MT1-MMP in RMS cells; however, it is likely that other known furin substrates are involved. Based on our findings and studies from other groups, we predict that furin inhibition will be of benefit for treatment of patients with RMS or other cancer types, particularly for prevention of metastasis.

5. Supplementary figures

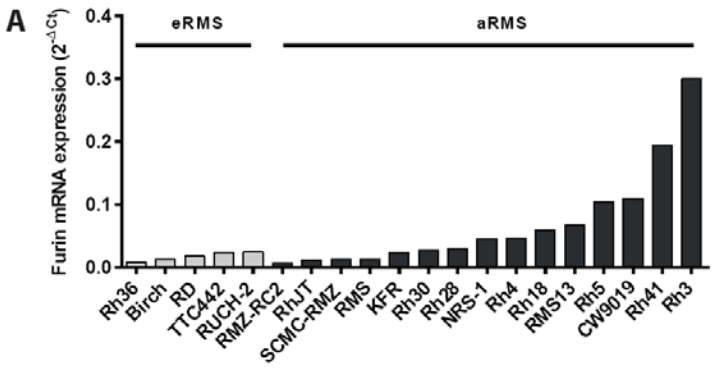

Figure S1. Furin mRNA levels in RMS cell lines. A) Endogenous furin mRNA levels were determined by qRT-PCR in 5 eRMS and 15 aRMS cell lines. Expression levels relative to GAPDH are shown.

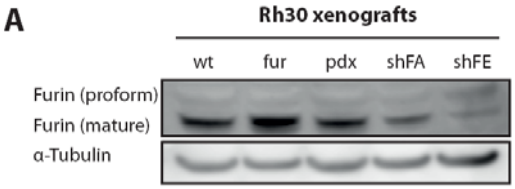

Figure S2. Furin protein levels in Rh30 xenografts. A) $5x10^6$ RMS cells with stable modulation of furin activity were s.c. injected in NOD/Scid IL2reg$^{-/-}$ mice and tumour growth was monitored over time. Tumours were excised upon a size of 750-1000 mm^3 and furin protein levels were assessed by immunoblot.

Chapter 3

The proprotein convertase furin is required to maintain viability of alveolar rhabdomyosarcoma cells

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Oncotarget (under revision)

This work was primarily done by Patricia Jaaks, who contributed the generation and validation of stable cell lines, proliferation, viability and apoptosis assays and tumour cell engraftments and tumour analyses. Gianmarco Meier (Master thesis) helped to establish stable cell lines and validated Rh30 cells. Nagjie Alijaj helped with the *in vivo* experiments. Eva Brack established Rh4 BAX and BAK double knockout cell lines. Peter Bode helped in the analysis of furin protein expression on tissue microarrays.

1. Introduction

Rhabdomyosarcoma (RMS) is the most common paediatric soft tissue sarcoma, representing 5-8% of all childhood cancers. RMS is subdivided in two main histological subgroups, embryonal (eRMS) and alveolar (aRMS) RMS. aRMS is more aggressive and is associated with a poorer prognosis with a 5-year survival of 48% (Ognjanovic, Linabery et al. 2009). Around 80% of aRMS tumours harbour the chimeric transcription factor PAX3/7-FOXO1 inducing a specific gene expression signature (Wachtel, Dettling et al. 2004; Davicioni, Finckenstein et al. 2006). A dominant role of PAX3/7-FOXO1 as oncogenic driver has been suggested (Sorensen, Lynch et al. 2002; Shern, Chen et al. 2014). Despite the dependence of aRMS tumours on PAX3/7-FOXO1 expression, transcription factors in general represent a challenging target.

Thus, many efforts have been made to characterise key pathways driving RMS progression in order to identify targets for novel therapeutic approaches. The insulin-like growth factor 1 receptor (IGF1R) is one among them. IGF1R is a transcriptional target of PAX3-FOXO1 (Ayalon, Glaser et al. 2001) and increased levels of the receptor correlate with poorer outcome in aRMS patients (Blandford, Barr et al. 2006). The ligand of IGF1R, the insulin-like growth factor 2 (IGF-2), is overexpressed in RMS (Gray, Tam et al. 1987) and acts as an autocrine mitogen (El-Badry, Minniti et al. 1990). Therefore, the IGF1R signalling pathway is a promising target to treat aRMS tumours. Different approaches to disrupt IGF1R signalling in RMS have been investigated: IGF1R anti-sense RNA (Shapiro, Jones et al. 1994), IGF1R-specific blocking antibodies (Kalebic, Tsokos et al. 1994) and the selective IGF1R inhibitor NVP-AEW541 (Scotlandi, Manara et al. 2005).

Proteolytic processing of precursor proteins by proprotein convertases (PCs) produces a large variety of bioactive proteins, such as growth factors, receptors, enzymes and cell-adhesion molecules. PCs are calcium-dependent serine proteases and seven of the nine family members (PC1, PC2, furin, PC4, PC5, paired basic amino acid cleaving enzyme 4 (PACE4) and PC7) process proproteins at basic residues. The other two, SK-1 and PCSK9, cleave after non-basic residues (Seidah and Prat 2012). Enhanced activity of PCs has been associated with pathological conditions like Alzheimer's disease (Bennett, Denis et al. 2000) and correlates with increased malignancy of certain cancer types such as prostate cancer, colon carcinoma or small cell lung carcinoma (Creemers, Roebroek et al. 1992; Khatib, Siegfried et al. 2001; D'Anjou, Routhier et al. 2011).

Furin was the first identified PC and acts within the constitutive secretory pathway (Molloy, Thomas et al. 1994). It processes many cancer-related proteins like IGF1R (Khatib, Siegfried et al. 2001), the vascular endothelial growth factor C (VEGF-C) (Siegfried, Basak et al. 2003) or the membrane-type 1 matrix metalloprotease 1 (MT1-MMP) (Remacle, Rozanov

et al. 2006). Aberrant furin expression is associated with neoplasias like head and neck cancer, breast, lung or colon cancers (Schalken, Roebroek et al. 1987; Cheng, Watson et al. 1997; Bassi, Mahloogi et al. 2001; Scamuffa, Siegfried et al. 2008). Upregulation of furin expression and translocation to the plasma membrane under hypoxic conditions are considered to favour invasiveness of cancer cells through enhanced proteolytic activation of MT1-MMP and TGFβ (McMahon, Grondin et al. 2005; Arsenault, Lucien et al. 2012). Thus, furin is a key activator of proproteins involved in cancer progression and represents a promising target to improve cancer treatment. Furin is endogenously inhibited by its own prodomain that is cleaved in a two-step autocatalytic activation process and the use of the prodomain as furin inhibitor has been proposed (Basak, Chen et al. 2010; Scamuffa, Sfaxi et al. 2014). Alternative inhibitory approaches are application of polyarginines (Kacprzak, Peinado et al. 2004), nanobodies (Zhu, Declercq et al. 2012) or α1-antitrypsin Portland (PDX) (Anderson, Thomas et al. 1993). However, most if not all proposed inhibitors lack selectivity for furin and are still in early developmental phases.

We previously identified specific RMS homing peptides that bind to the proprotein convertase (PC) furin (Hajdin, D'Alessandro et al. 2010). Here, we investigate in detail the role of furin in aRMS progression *in vitro* and *in vivo*. We use inducible expression of furin specific shRNA to decrease furin expression and activity. Furin silencing resulted in impaired cell viability, caspase-dependent apoptosis and regression of tumours. We further found that furin is expressed in most patient biopsies. Therefore, we propose the proprotein convertases furin as novel target for aRMS therapy.

2. Material and methods

Ethics Statement

All animal experiments were approved and monitored by the veterinary office of the Canton of Zurich according to the Swiss Federal Law.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Data are expressed as mean ± standard deviation (SD). Statistical significance was tested for multiple comparison with analysis of variance (ANOVA). The differences were considered to be significant if $p<0.05$.

Cell lines and cell culture

The cell lines Rh4, Rh30 (Peter Houghton, St. Jude Children's Hospital, Memphis, TN), Rh3 (Susan Ragsdale, St. Jude Children's Hospital, Memphis, TN), CW9019 (Soledad Gallego, Hospital Universitari Vall d'Hebron, Barcelona, Spain), MRC5 and HEK293T (ATCC, LGC Promochem) were cultured in DMEM (Sigma-Aldrich), supplemented with 100 U/mL penicillin/streptomycin, 2 mM L-glutamine, and 10% FBS (Life Technologies) in 5% CO_2 at 37 °C. aRMS cell lines were tested and authenticated by cell line typing analysis (STR profiling) on 2011/2014 and positively matched (Hinson, Jones et al. 2013).

Production of lentiviral particles and transduction of cells

Third generation lentiviral packaging plasmids and vectors containing shRNA (scrambled (scri) or targeted against furin (target sequence shFAi: CCTGTCCCTCTAAAGCAATAA; target sequence shFEi: CCACATGACTACTCCGCAGAT)) under a U6-tet promoter as well as reporter genes GFP and puromycine resistance under PGK promoter were purchased from Cellecta (Cellecta Inc. Mountain View, USA). Lentiviral particles were generated by calcium phosphate-based transfection of HEK293T cells. Transducing units per mL (TU/mL) were determined through titration of lentiviral particles on Rh4 cells and analysis of % GFP positive cells by fluorescent activated cell sorting (FACS) on a FACSCanto II (BD Bioscience, Missisagua, Canada) 48h post transduction. TU/mL were determined using the formula $TU/mL= (F*C*D)/V$ (F=frequency of GFP positive cells; C=number of cells; D=virus dilution; V=volume of inoculum). Cells were transduced with a multiplicity of infection (MOI) of 5. Transduction was performed by addition of required amount of lentiviral particles in DMEM in the presence of 10 µg/mL polybrene (Sigma-Aldrich) followed by centrifugation (32 °C, 1h, 800 g). Medium was replaced with fresh medium 4h post transduction start and efficiency of transduction was determined by analysis of GFP-positive

cells by FACS 7-10 days post transduction. Transduction efficiency was over 98% for all cell lines.

To generate Rh4 cells with double knock out for BAX and BAK a CRISPR/Cas9-based approach was chosen. Vectors based on the LentiCrispr v1 vector (Addgene) were modified to carry a TagBFP for selection and delivered packaged in lentiviral particles. Two different sgRNAs for each target gene were evaluated individually for their knock down efficiency by immunoblot and the two best sequences were then co-delivered to achieve a double knock out (Rh4 BAX/BAK; sgRNA BAX: CGAGTGTCTCAAGCGCATCG; sgRNA BAK: ACGGCAGCTCGCCATCATCG). A scrambled sgRNA sequence was used as control (Rh4 sc; sgRNA sc: GCACTACCAGAGCTAACTCA).

qRT-PCR

Total RNA was extracted from cells or tumour tissue using the Qiagen RNeasy Kit (Qiagen, Hombrechtikon, Switzerland) and reverse-transcribed using oligo (dT) primers and Omniscript reverse transcriptase (Qiagen). qRT-PCR pas performed for furin (Hs00965485_g1), PC1 (Hs01026107_m1), PC2 (Hs0015992_m1), PC4 (Hs00399493_m1), PC5 (Hs00196400_m1), PACE4 (Hs00159844_m1), PC7 (Hs00237114_m1), S1P (Hs00921626_m1) and PCSK9 (Hs00545399_m1) employing TaqMan gene expression master mix (all Life Technologies). Cycle threshold (C_T) values were normalised to GAPDH (Hs02758991_g1) or HMBS (Hs00609296_g1) for cells or tumour tissue, respectively. Relative expression levels were calculated using the $\Delta\Delta C_T$ method.

Immunoblot

Total protein extracts from cells or tumour tissue were separated using 4-12% Bis-Tris SDS-PAGE gels (Life Technologies) and transferred to nitrocellulose membranes (PROTAN; Schleicher & Schuell). Membranes were blocked with 5% milk powder in TBS/0.05% Tween and subsequently incubated with primary antibodies overnight at 4°C. After washing in TBS, membranes were incubated with IgG horseradish peroxidase (HRP)-linked secondary antibody for 1h at RT. Proteins were detected using ECL detection reagent (Fisher Scientific) after washing in TBS.

IGF1 stimulation

RMS cells were treated with 25 ng/mL DOX for a total of 48h. After 24h cells were put on starvation medium (DMEM, 0.2% FBS). Cells were stimulated for 10 min with 50 ng/mL IGF1 (ab73455, Abcam). Cells were washed with ice-cold PBS and snap frozen in liquid nitrogen. Whole cell lysates were prepared as described above and IGF1Rβ, Akt, phosphorylated Akt, Erk1/2 and phosphorylated Erk1/2 were analysed by immunoblotting.

Antibodies

The following antibodies were used: anti-furin MON-152 (1:750, ALX-803-017-R100, Alexis Corporation), anti-IGF1Rβ (9750S, Cell Signaling), anti-caspase-9 (9502, Cell Signaling), anti-cleaved caspase-7 (9491, Cell Signaling), anti-PARP (9542, Cell Signaling) and anti-α-tubulin (1:5000, A5316, Sigma-Aldrich). All Cell Signaling antibodies were used in a dilution of 1:1000.

Cell proliferation and viability assays

Cells were seeded in a 96-well format in 100 µL medium. To assess metabolic activity 10 µL WST-1 reagent (Roche) were added. After 30-60 min incubation at 37°C in the dark absorbance was measured and recorded as $\Delta OD_{440-640}$. Background values were subtracted. To determine their number, cells were fixed in 4% paraformaldehyde for 10 min at RT and stained in 0.05% crystal violet (Sigma-Aldrich) for 20 min at RT. Excess crystal violet was removed and cells air dried. Dye was dissolved in 100% methanol and absorbance was measured at OD_{594}. Cell proliferation was determined through BrdU incorporation (Cell Proliferation ELISA, BrdU (Chemiluminescent), Roche) according to manufacturer's instructions. Cells were labelled with BrdU for 2h at 37°C, fixed and stained for 1h with anti-BrdU-POD solution. Chemiluminescent substrate was added and luminescence measured.

Caspase 3/7 activity

Cells were seeded in a 384-well format in 20 µL medium. At the desired time point, 10 µL Caspase Glo® 3/7 Substrate (Promega) were added and plates vigorously shaken for 1 min. After 30 min incubation at RT in the dark luminescence was measured.

Cell cycle distribution

One million cells were collected and fixed in ice cold 70% ethanol at -20°C for at least 2h. Fixed cells were washed in PBS and resuspended in propidium iodide (PI) solution (20 µg/mL PI and 200 µg/mL RNase A (Sigma-Aldrich) in PBS/0.1% Triton X-100). PI signal was assessed by FACS. Cell cycle distribution was analysed with FlowJo software, version 10 (TreeStar).

Immunoprecipitation of active BAX

For detection of active BAX, cells were lysed in CHAPS lysis buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 1% CHAPS) as previously described (Häcker, Dittrich et al. 2009). Briefly, 1000 µg protein were incubated overnight at 4°C with 5 µg5 mouse anti-BAX antibody (6A7, Sigma) followed by addition of 10 µL Dynabeads Protein G (Invitrogen) and further incubation for 2h at 4°C. Dynabeads were washed with lysis buffer and BAX levels were analysed by immunoblotting using rabbit anti-BAX (1:1000; 5023, Cell Signaling).

Xenograft studies

To study *in vivo* growth $2.5x10^6$ Rh30 or $3.25x10^6$ Rh4 cells were engrafted subcutaneously in 6-week-old NOD/SCID IL2rg$^{-/-}$ mice (female, 20-25 g; Charles River). In

Rh30 xenografts doxycycline (DOX)-supplemented food (SynergyHealth) was given one week prior engraftment. DOX administration in Rh4 xenografts was started upon a tumour size of 200-250 mm^3 through intraperitoneal (i.p.) injection of 0.053 mg DOX/g body weight on two consecutive days and continuous DOX-supplemented food. Control treated mice were injected with PBS. Body weight and tumour growth were monitored over time. Tumour volumes were determined by measuring two diameters (d1, d2) in right angles using a digital caliper ($V = (4/3)\ \pi\ r^3$; $r = (d1 + d2)/4$). Upon reaching a tumour size of 1000 mm^3 mice were sacrificed, tumour tissue was harvested and snap frozen for RNA and protein extraction.

Immunohistochemistry

Sections (3 µm thick) of formalin-fixed, paraffin-embedded tissue were mounted on glass slides (SuperFrost Plus; Menzel), deparaffinised, rehydrated and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed on Leica BondMax instruments using Refine HRP-Kits (Leica DS9800; Leica Microsystems Newcastle, Ltd.). Dewaxed and pretreated (Epitop Retrieval Buffer 2, Leica Biosystems, Muttenz, Switzerland; 60 min, 100°C) paraffin slides were incubated with anti-cleaved caspase-3 antibody (1:500; #9661, Cell Signaling).

Tissue microarray

A tissue microarray with 248 cores from 124 RMS tumours (17 aRMS with known *FOXO1* gene rearrangements and 107 eRMS) was constructed. Tumours used were collected at the University Hospital Zurich, Switzerland and at the Kiel Paediatric Tumour Registry, Germany. Immunohistochemistry was performed essentially as described above. Paraffin slides were dewaxed, pretreated (Epitop Retrieval Buffer 2, Leica Biosystems; 10 min, 95°C) and incubated with furin antibody (1:3000; Ab28547, Abcam). Overall staining was graded according to intensity and assigned to four different groups (no expression, low, intermediate or high expression). Tissue microarrays were analysed double-blinded.

3. Results

Validation of inducible furin silencing in alveolar RMS cells

In order to simulate specific furin inhibition we generated stable aRMS cell lines with tetracycline-dependent expression of furin shRNA (shFAi and shFEi) or control shRNA (scri) (Figure 1A). Furin silencing was assessed 24-72h after doxycycline (DOX) treatment. Upon DOX treatment furin mRNA was reduced to around 15% in Rh30 cells (Figure 1B) and to 6.5-25% in other aRMS cell lines (Figure S1A).

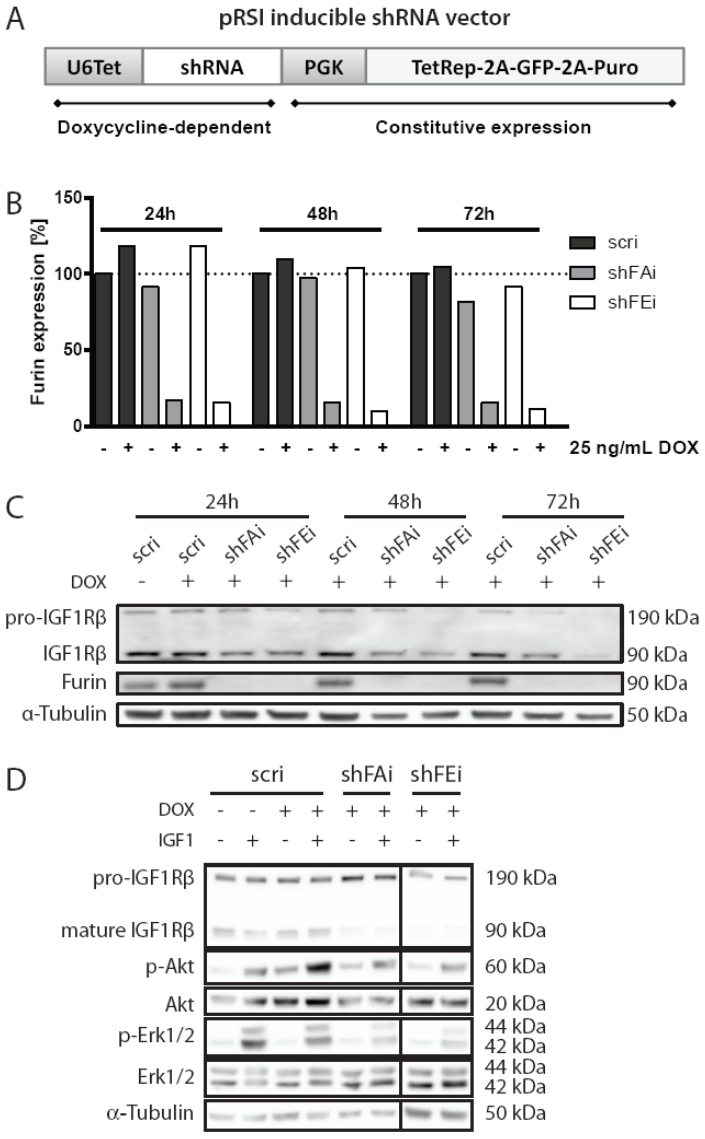


Figure 1. Inducible silencing of furin in alveolar RMS cells.
A) Schematic overview of the pRSI vector for inducible shRNA expression. Tet repressor, GFP and puromycin resistance under control of a phosphoglycerate kinase 1 (PGK) promoter are constitutively expressed. The tetracycline dependent promoter (U6Tet) controls expression of shRNA. Rh30 cells transduced with pRSI coding for control (scri) or furin (shFAi, shFEi) shRNAs were treated with 25 ng/mL doxycycline (DOX). B) Furin mRNA levels were determined in Rh30 cells by qRT-PCR at different time points after DOX addition. Expression levels relative to GAPDH and normalized to non-treated control (scri) cells are shown. The experiments were performed in duplicates. C) Protein expression of furin and its substrate IGF1Rβ were examined in Rh30 cells by immunoblotting 24h, 48h and 72h post-induction. One representative experiment is shown. D) Rh3 cells were stimulated for 10 min. with 50 ng/mL IGF1 48h post induction of furin silencing and phosphorylation of IGF signalling pathway mediators Akt and Erk1/2 was examined by immunoblotting. One representative experiment is shown.

Subsequently, we analysed furin protein levels in Rh30 cells by immunoblotting und could confirm that furin protein levels were strongly reduced upon DOX treatment (Figure 1C). To monitor furin activity we analysed the maturation of a known furin substrate, IGF1Rβ. Decrease of furin led to clear reduction of mature IGF1Rβ, indicating that furin activity is

indeed lowered upon silencing of furin (Figure 1C). mRNA levels of other PCs, examined by qRT-PCR after 48h of treatment with 25 ng/mL DOX, were unaffected suggesting that furin silencing was specific and was not compensated for by increased mRNA expression of other PCs (Figure S1B). In conclusion, we generated and validated aRMS cell lines with functional furin silencing that is inducible by application of DOX. Furthermore, effects of furin silencing on IGF1R processing and downstream Akt activation upon stimulation with IGF1 could be demonstrated in Rh3 cells (Figure 1D) and to a lesser extent in Rh4 cells (Figure S1C). This suggests that activation of the IGF signalling pathway is furin dependent at least in a subset of aRMS cells.

Furin silencing decreases cell viability and proliferation

Furin processes a variety of substrates, e.g. IGF1R, that support cancer cell viability and proliferation. Thus, we induced furin silencing in four different aRMS cell lines, Rh30, Rh4, CW9019 and Rh3 and assessed cell viability. Cell viability was clearly decreased, as compared to non-treated cells (Figure 2A- Rh30: 27%, Rh4: 7%; Figure S2A- CW9019: 48%, Rh3: 19%). The number of viable adherent cells was reduced up to 5-fold compared to non-treated cells (Figure 2B- Rh30: 23%, Rh4: 20%; Figure S2B- CW9019: 54%, Rh3: 23%). Furthermore, proliferation, examined by BrdU incorporation, was much lower in furin silenced Rh30 and Rh4 cells than in cells expressing control shRNA and went down to 48% and 15%, respectively (Figure 2C). We also noted a slight toxic effect upon expression of the control shRNA (scri) in all aRMS cell lines (Figure 2A-C). Since DOX application alone had no effect on viability of aRMS wt cells (data not shown), we assume that this slight effect may be due to high shRNA expression after DOX treatment. Furthermore, we investigated furin silencing in MRC5 fibroblast cells and found that induced silencing of furin had no significant impact on viability of MRC5 fibroblast cells (Figures 2D and E). Thus, furin activity seems to be a critical factor in viability and proliferation of aRMS cells, whereby a decrease of furin does not alter cell viability of normal fibroblasts.

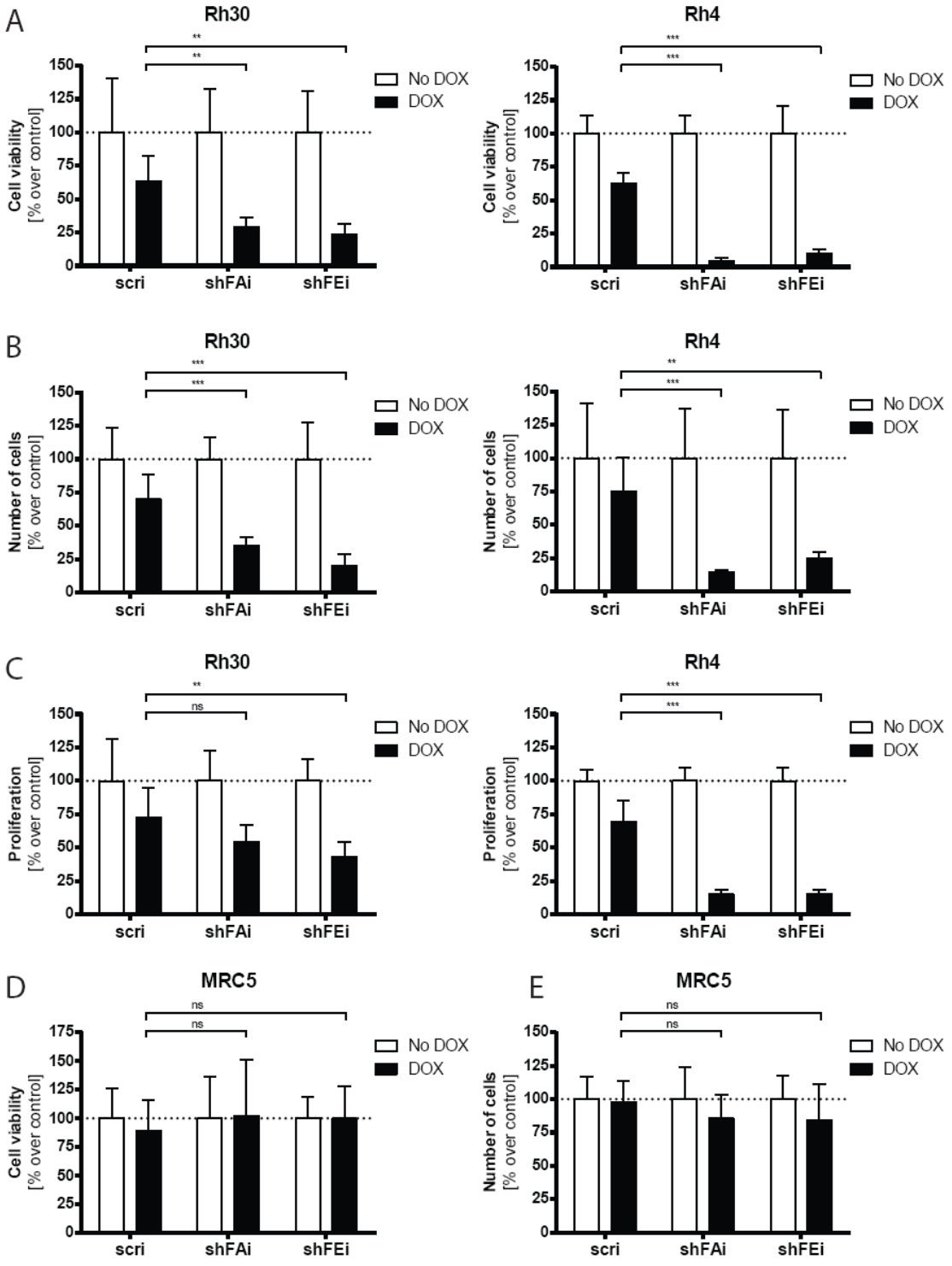


Figure 2. Cell viability and proliferation of aRMS cells are decreased upon furin silencing. Rh30, Rh4 and MRC5 cells were treated with 25 ng/mL DOX for 96h and analysed. A) Cell viability in Rh30 and Rh4 cells was determined with a WST-1 assay. B) The number of Rh30 and Rh4 cells was quantified by staining with crystal violet. C) Proliferation in Rh30 and Rh4 cells was measured by incorporation of BrdU. D) Cell viability in MRC5 cells was determined with a WST-1 assay. E) The number of MRC5 cells was quantified by staining with crystal violet. All data represent mean ±SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.005, two-way ANOVA.

Reduced furin activity results in caspase-dependent cell death involving mitochondria

In our initial investigation of aRMS cell viability upon furin silencing we observed that reduced furin activity not only impaired viability and proliferation of the cells, but also led to morphological changes and cell death in Rh4 and Rh3 cells. Therefore, we examined the cell cycle distribution of Rh4 and Rh3 cells 72h after DOX treatment and found a clear increase of cells in sub-G1 phase (Figure 3A- shFAi: 54-62%, shFEi: 26-32%). As a positive control for apoptosis we treated aRMS cells with a combination of 1 µM of the IGF1R-inhibitor AEW451 (AEW) and 275 nM of the dual PI3K/mTOR inhibitor BEZ235 (BEZ) for 24h, conditions that induce apoptosis in aRMS cell lines (Wachtel, Rakic et al. 2014). As expected, treatment with BEZ/AEW led to an increase of cells in sub-G1 phase (Figure 3A- Rh4: 22%, Rh3: 18%). To assess the contribution of caspases in cell death induction we treated the cells with 100 µM of the pan-caspase inhibitor zVAD. Cell cycle profiles of furin silenced or BEZ/AEW treated cells were restored in the presence of zVAD similar to non-treated levels, indicating that induced cell death is caspase dependent (Figure 3A).

To further elucidate the mechanism underlying cell death, we analysed cleavage of caspase-7 and -9 and PARP by immunoblotting. In Rh4 and Rh3 cells both caspases as well as PARP were cleaved upon furin silencing and BEZ/AEW treatment, confirming apoptosis induction. Cleavage of the effector caspase-7 and of PARP could be almost completely prevented in the presence of zVAD, whereby inhibition of caspase-9 cleavage was incomplete (Figure 3B). Caspase activity of the caspases-3 and -7 was elevated 5 to 7-fold upon furin silencing in Rh4 and Rh3 (Figure 3C and data not shown), but not in Rh30 or CW9019 cells (data not shown). This suggests that in some aRMS cells furin silencing mainly affects proliferation and viability, whereas in other aRMS cell lines a decrease in furin activity additionally induces caspase-dependent cell death. Furthermore, application of zVAD to Rh4 cells upon induction of furin silencing only partially rescued cell viability as assessed by measuring metabolic activity and number of attached cells (Figure S3A and B). In this case caspase inhibition prevented apoptosis, but did not prevent anti-proliferative processes.

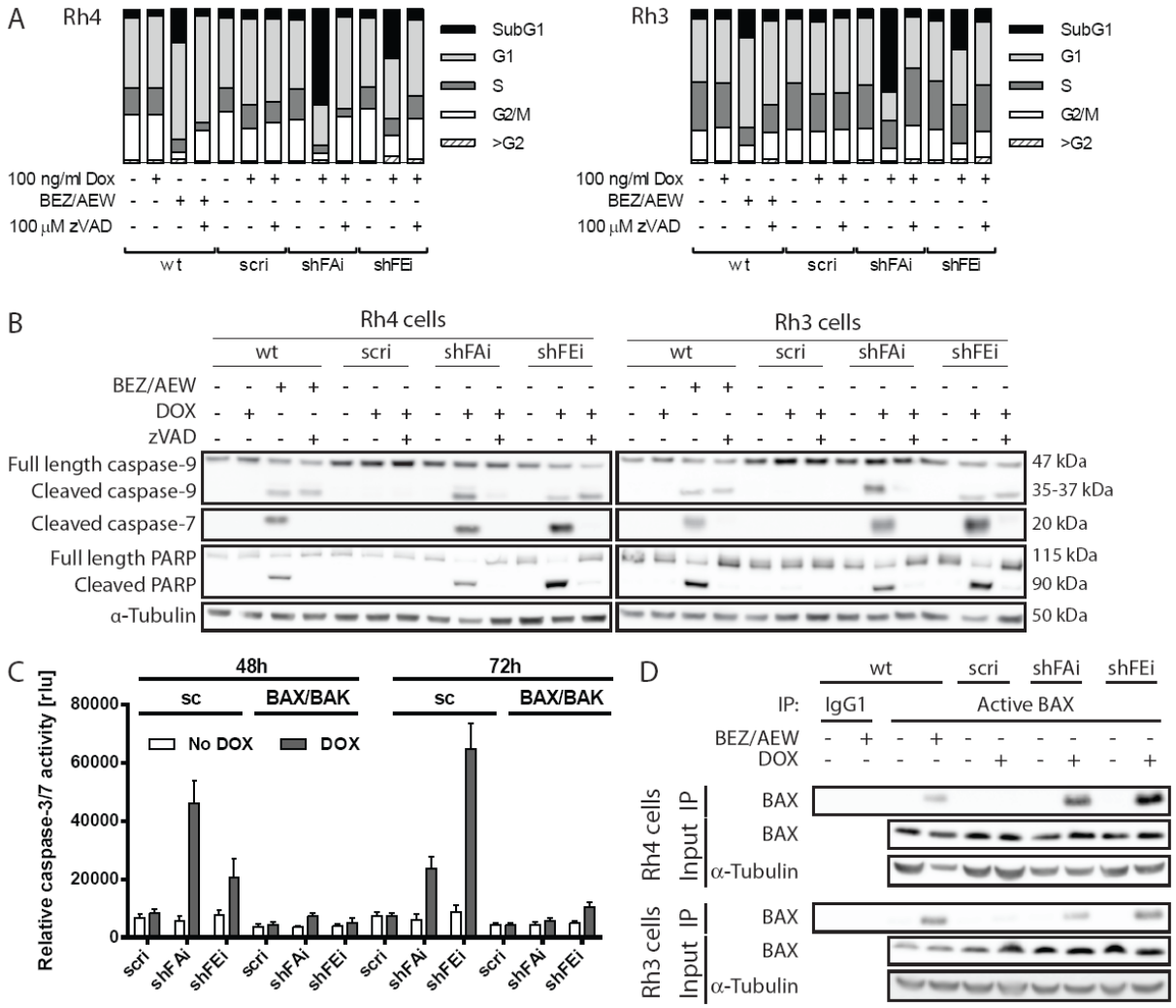


Figure 3. Cell death induction upon furin silencing is caspase dependent and involves mitochondria. A) Caspase inhibition restores normal cell cycle distribution. Rh4 and Rh3 cells were treated with 100 ng/mL DOX for 72h in the presence or absence of 100 μM zVAD, fixed and stained with propidium iodide and cell cycle distribution was studied by FACS. B) Inhibition of caspase activity rescues cleavage of caspase-7 and PARP, but not caspase-9 cleavage. Furin silencing was induced in Rh4 (left panel) and Rh3 (right panel) cells by addition of 100 ng/mL DOX in the presence or absence of zVAD, total protein was extracted 72h post induction, and cleavage of caspase-9 and -7 and PARP was analysed by immunoblotting. Wt cells were treated with 275 nM BEZ235 and 1 μM AEW451 for 24h as positive control (A-B, D). C) Effector caspase activation is mitochondria dependent. Rh4 cells with a stable BAX and BAK (BAX/BAK) or control (sc) knock out were generated using a lentiviral CRISPR/Cas9 based approach. Knock out cells were stably transduced with inducible furin silencing constructs. Cells were treated with 100 ng/mL DOX for 48 and 72h and caspase-3/7 activity was analysed by a caspase specific luminescent assay. Data represent mean ±SD of three independent experiments. D) Apoptosis induction is dependent on BAX activation. Rh4 and Rh3 cells were treated with 100 ng/mL DOX for 48h (scri, shFAi) or 72h (shFEi) and active BAX was immunoprecipitated using a conformation-specific antibody.

Furin silencing results in remission of Rh4 xenografts *in vivo*

Reduced furin activity decreased cell viability and proliferation in Rh4 cells and further induced caspase-dependent apoptosis *in vitro*. To examine the role of furin in Rh4 tumour growth we engrafted 3.25 million cells harbouring either inducible furin shRNA (shFAi and shFEi) or control shRNA (scri). In order to simulate a potential therapy aiming at abrogating furin activity in established tumours, mice were treated with DOX once tumours reached a size of 200-300 mm^3 (Figure 4A). Mice were sacrificed upon reaching a tumour size of 1000 mm^3. PBS-treated tumours took on average 16-17 days to attain this size (Figure 4B- scri: 17 days; shFAi: 16.6 days; shFEi: 16.5 days). Rh4 scri tumours treated with DOX showed slightly decelerated tumour growth and took on average 25 days to reach a size of 1000 mm^3. In contrast, Rh4 shFAi and shFEi tumours went into full remission after 10-15 days. 4 out of 5 Rh4 shFAi tumours relapsed 9-20 days later, whereby the last tumour of this group never relapsed, not even after DOX withdrawal 60 days post remission. None of the Rh4 shFEi DOX-treated mice relapsed (Figure 4B). Assessment of furin mRNA by qRT-PCR revealed that only half of Rh4 shFAi DOX-treated relapsed tumours maintained low levels of furin. The other two tumours showed higher furin mRNA levels (41% and 75% over control treated Rh4 shFAi tumours), indicating a possible outgrowth of cells with ineffective furin silencing (Figure 4C). To further investigate the mechanisms leading to remission of Rh4 shFAi and shFEi tumours upon DOX administration, we sacrificed few mice 5 days after initial administration of DOX. Decreased furin mRNA levels were confirmed by qRT-PCR (Figure S5- shFAi: around 30%; shFEi: 15%). Total protein was extracted from tumour tissue and protein levels of different cell death markers were examined by immunoblotting. The three tumours with lower furin levels displayed elevated levels of cleaved caspase-9 and -7 and cleaved PARP as compared to Rh4 scri tumours (Figure 4D). Furthermore, Rh4 shFEi, but especially shFAi tumours showed high levels of cleaved caspase-3 after 5 days of DOX treatment (Figure 4D). Expression of scri in control tumours did not lead to activation of this effector caspase.

Thus, we could show that induced silencing of furin leads to full remission of Rh4 tumours *in vivo*. This is most likely achieved through initiation of apoptotic processes, as shown by activation of key caspases. Beside reactivation of furin expression, other, yet undetermined, processes might lead to relapse of tumours and should be further investigated.

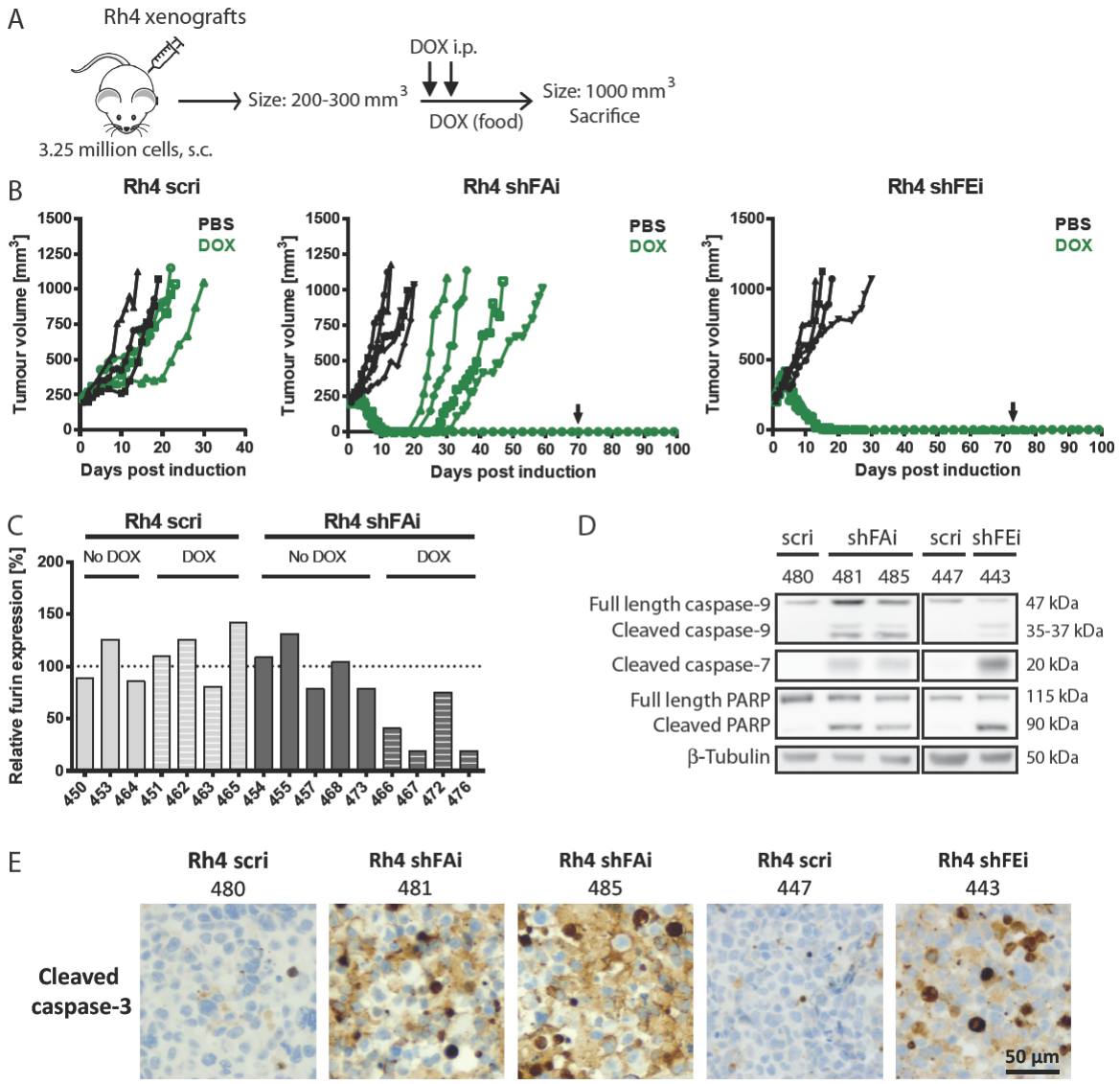


Figure 4. Induction of furin silencing results in remission of Rh4 tumours in vivo. A) Schematic overview of Rh4 xenograft model. 3-5 NOD/SCID mice per group were engrafted s.c. with 3.25 million Rh4 cells. Expression of shRNA was induced through administration of DOX (i.p. injection of 53 mg/kg DOX on two consecutive days combined with DOX-supplemented food) once a tumour size of 250-300 mm3 was reached. Mice were sacrificed upon a tumour size of 1000 mm3. B) Tumour growth over time as monitored by caliper measurements in Rh4 xenografts. Arrows indicate that mice were put back on normal food approximately 60 days post onset of remission. C) Furin mRNA levels in Rh4 tumours. Tumour tissue was collected, total mRNA isolated and furin mRNA levels analysed by qRT-PCR. D-E) Apoptotic markers in Rh4 tumours after 5 days of DOX treatment. D) Caspase-9, cleaved caspase-7 and PARP levels were examined by immunoblotting. E) Levels of cleaved caspase-3 were analysed by immunohistochemistry.

Tumour growth of Rh30 xenografts *in vivo* is delayed after furin silencing

Induction of furin silencing diminished proliferation and cell viability of Rh30 cells *in vitro*. To determine the impact of furin activity on Rh30 tumour growth *in vivo* we engrafted 2.5 million Rh30 cells with inducible furin shRNA and shRNA expression was induced by administration of DOX-supplemented food 7 days prior engraftment of tumour cells (Figure 5A). Control groups were fed normal food. Tumour growth was monitored over time through calliper measurements and mice were sacrificed once tumours reached a size of 1000 mm^3. Rh30 scri tumour growth was similar with or without DOX supplement and tumours reached a size of 300 mm^3 after 25 days. Conversely, early tumour growth was clearly delayed in Rh30 shFAi and shFEi tumours in the presence of DOX and tumours took approximately 50% more time to reach 300 mm^3 (Figure 5B- shFAi- no DOX: 21 days, DOX: 31days; shFEi- no DOX: 22 days, DOX: 34 days). To investigate furin silencing efficiency we extracted RNA from tumour tissue and evaluated furin mRNA levels by qRT-PCR. Furin mRNA levels of Rh30 shFAi or shFEi tumours with DOX as compared to respective tumours without DOX were on average 15% and 11%, respectively (Figure 5C), confirming effective furin silencing. Despite having low furin levels, once furin silenced Rh30 tumours exceeded a size of approximately 300 mm^3, they grew almost as fast as control tumours. However, a clear delay in early tumour growth could be observed, suggesting that furin activity is important in early phases of Rh30 tumour growth *in vivo*.

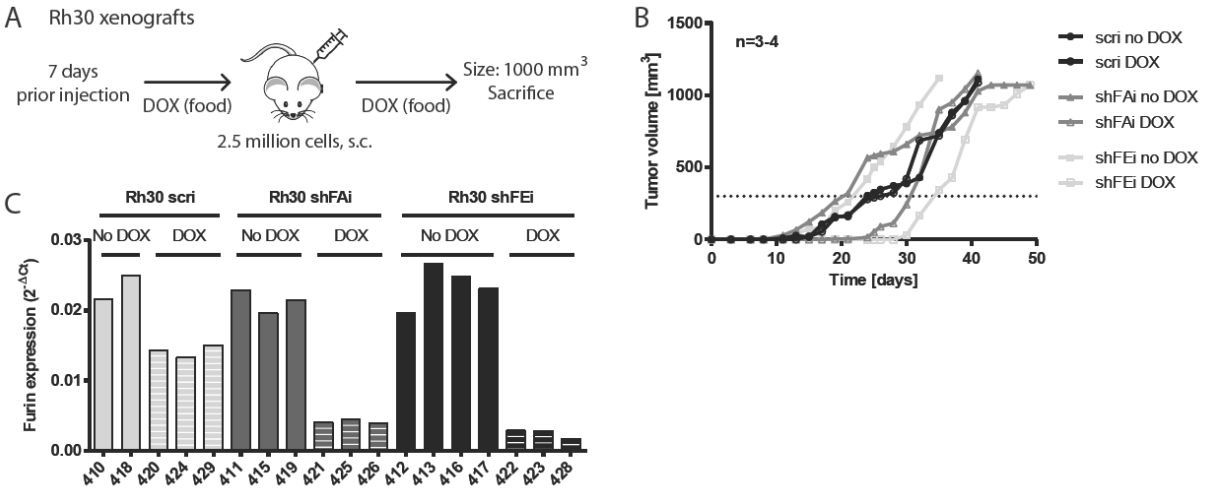


Figure 5. Decrease of furin activity delays tumour growth of Rh30 xenografts *in vivo*. A) Schematic overview of Rh30 xenograft model. 3-4 NOD/Scid mice per group were fed with or without DOX-supplemented food starting 7 days prior engraftment. Mice were then engrafted s.c. with 2.5 million Rh30 cells. Tumour growth was monitored over time and mice were sacrificed once tumours reached a size of 1000 mm^3. B) Absence of furin activity delays initial tumour growth. Tumour growth rate was monitored by caliper measurements. Data represent mean tumour size of 3-4 mice per group. C) Furin expression is reduced upon induction of furin specific shRNA. Tumour tissue was collected upon sacrifice of mice, total mRNA isolated and furin mRNA levels analysed by qRT-PCR. Expression levels relative to HMBS are shown.

Most RMS biopsies express furin

To evaluate the potential of furin targeted therapies in patients we examined furin protein expression in 89 RMS biopsies (74 eRMS, 15 aRMS). A TMA was stained for furin and expression was graded according to overall intensity of the staining in tumour cells (Figure 6A). We found that, independent of the subtype, over 86% of RMS tumours expressed low to high amounts of furin (Figure 6B), whereby most tumours showed low to intermediate expression. A correlation between the level of furin protein in tumours and event-free or overall survival of the patients could not be confirmed (data not shown).

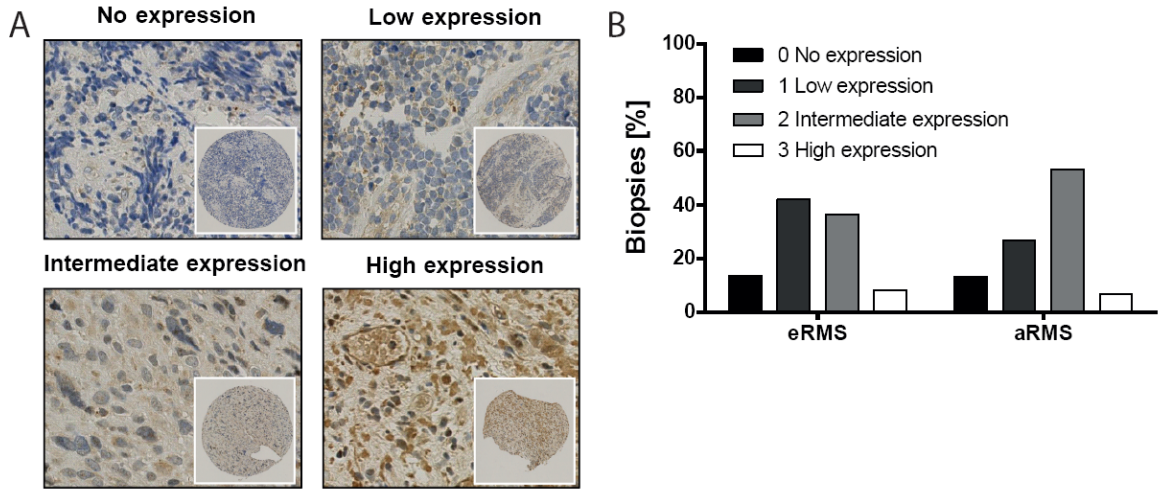


Figure 6. Furin is expressed in most RMS biopsies. A patient-derived tissue microarray was immunohistochemically stained for furin (n=89). Patients were grouped according to no expression (n=12), low (n=35), intermediate (n=35) and high (n=7) furin expression. A) Representative pictures of biopsies from all four categories. B) Distribution of furin expression intensity based on RMS subtype. Furin expression levels were analysed for 74 eRMS and 15 aRMS biopsies.

4. Discussion

This study establishes for the first time a link between furin activity and rhabdomyosarcoma progression both *in vitro* and *in vivo*. Silencing of furin led to significantly decreased cell viability and induction of caspase-dependent apoptosis *in vitro*. Furthermore, depletion of furin activity *in vivo* resulted in regression or delayed tumour growth of aRMS tumours.

Furin is known to process many growth factors and their receptors, like IGF-1 and 2, IGF1R or transforming growth factor β (TGFβ) (Lehmann, André et al. 1998; Tian, Huang et al. 2011). Accordingly, silencing of furin led to significant reduction of cell viability and proliferation rate in all investigated aRMS cell lines. Furin silencing had no effect on MRC5 fibroblast viability, suggesting that aRMS, but not normal cells are dependent on furin activity. A subset of the aRMS cell lines tested was particularly sensitive to furin silencing and underwent BAX/BAK- and caspase-9-dependent apoptosis. This indicates that furin silencing primarily triggers the intrinsic apoptosis pathway. Others have shown that disruption of the IGF1R and/or PI3K/mTOR pathways leads to apoptosis in Rh3 and Rh4 cell lines (Scotlandi, Manara et al. 2005; Wachtel, Rakic et al. 2014). In this study we found evidence that activation of the IGF signalling pathway is dependent on furin activity. We therefore hypothesize that incomplete activation of growth factor-dependent pathways is the principal cause of cell death induced upon furin silencing in sensitive cell lines. It remains to be investigated which proapototic BH3-only proteins are involved, but treatment of Rh3 and Rh4 cells with a combination of the IGF1R inhibitor AEW541 and the dual PI3K/mTOR inhibitor BEZ235 results in BMF-dependent apoptosis (Wachtel, Rakic et al. 2014). Other proapototic BH3-only proteins linked to growth factor deprivation are BAD, BIM and PUMA. For instance, phosphorylation of BAD through active protein kinase Akt results in binding and sequestration by 14-3-3 scaffold proteins (Zha, Harada et al. 1996; del Peso, Gonzalez-Garcia et al. 1997). Thus, lower levels of activated Akt due to decreased IGF signalling upon furin silencing might promote release of BAD and mitochondria pore formation. Further investigations will be required to elucidate the exact mechanism of cell death after silencing of furin.

To examine the impact of furin silencing on aRMS tumour growth *in vivo* we engrafted two aRMS cell lines, Rh30 and the sensitive Rh4. In Rh30 xenografts, upon furin silencing, we observed delay of early tumour growth, suggesting that furin cleaves one or more precursor proteins that are crucial for early steps of aRMS tumour growth after xenotransplantation.

A signalling pathway likely to be involved is the VEGF signalling axis. Gee et al. confirmed expression of VEGF, VEGFR1, which is a transcriptional target of PAX3-FOXO1 (Barber, Barber et al. 2002), and VEGFR2 in aRMS cell lines and were the first to propose a

role of VEGF signalling in RMS growth (Gee, Tsuchida et al. 2005). Processing of precursor VEGF-C and VEGF-D through furin allows their binding to VEGFR2 and induction of vasculogenesis and angiogenesis (Millauer, Wizigmann-Voos et al. 1993; Joukov, Sorsa et al. 1997; Siegfried, Basak et al. 2003; McColl, Paavonen et al. 2007). Additionally, only furin-processed VEGF-C induces tumourigenesis in mice (Siegfried, Basak et al. 2003), highlighting the importance of PC activity in the VEGF signalling axis. Therefore, reduced VEGF-C and VEGF-D processing upon furin silencing might suppress proper formation of angiogenic and lymphangiogenic vessels inside the new tumours and decrease VEGFR2-dependent aRMS tumour cell growth.

When furin was silenced in established Rh4 tumours, regression was observed. Apoptosis was induced, as confirmed by activation of caspases-9, -3 and -7 and PARP cleavage. Four of the Rh4 shFAi tumours relapsed and furin re-expression was found in two of them. Since we used a heterogeneous cell pool, it is likely that outgrowth of a subset of non-silenced cells caused the relapse. Further investigations will be required to uncover the underlying mechanisms of tumour regrowth.

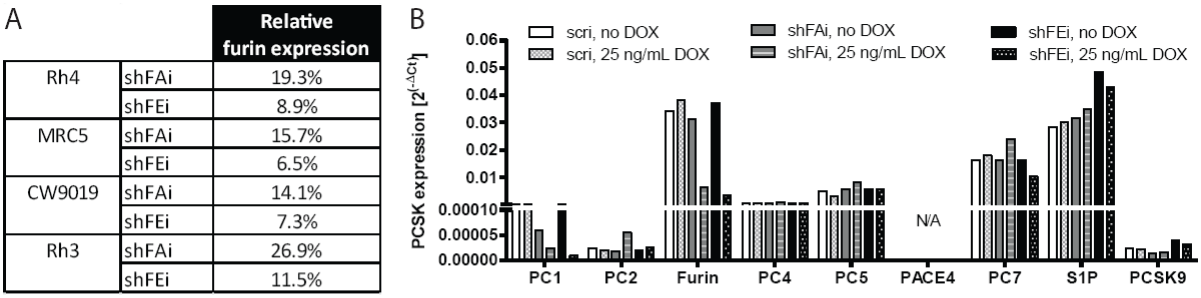
We found furin expression in over 86% of tested aRMS and eRMS biopsies, but no significant correlation could be confirmed between furin expression and event-free or overall survival. It is possible that other risk-factors interacted with the analysis of furin influence on survival, or that furin expression alone is not a good marker to for its role in cancerogenesis, but rather its subcellular localization and activity are. Our results are in line with other studies demonstrating the role of furin and other PCs for cancer cell survival (D'Anjou, Routhier et al. 2011; Scamuffa, Sfaxi et al. 2014; Sfaxi, Scamuffa et al. 2014). Thus, PCs are emerging targets for the treatment of cancer and development of specific inhibitors will be of central importance. Levesque et al. recently reported the improvement of peptide-based specific PACE4 inhibitors for treatment of prostate cancer (Levesque, Fugere et al. 2012; Levesque, Couture et al. 2015) and Zhu et al. described the first furin-specific inhibitory nanobodies (Zhu, Declercq et al. 2012). Despite their great clinical potential, specific inhibitors of furin and furin-like PCs are still in early phases of development. Besides, the safety of such inhibitors will have to be closely examined as most PCs are widely expressed in human tissues. Nevertheless, disruption of furin activity has been used successfully to enhance an anti-tumour immune response in patients with advanced cancers. Safety and efficient immune response of an autologous tumour cell vaccine (FANG) that combines furin shRNA and expression of GM-CSF were confirmed and benefits of treatment were observed from phase I trials in Ewing sarcoma and hepatocellular carcinoma patients (Senzer, Barve et al. 2012; Nemunaitis, Barve et al. 2014; Ghisoli, Barve et al. 2015). Additionally, increased presence of furin on the cell surface of tumour cells could be used for targeted delivery of

chemotherapeutics, as we could show previously that furin is the target receptor for RMS-homing peptides (Hajdin, D'Alessandro et al. 2010). In conclusion, our data clearly validate furin as a novel target in aRMS and we predict that the majority of paediatric RMS patients would benefit from inhibition of furin activity, either alone or in the context of immune therapy.

5. Supplementary figures

A

		Relative furin expression
Rh4	shFAi	19.3%
	shFEi	8.9%
MRC5	shFAi	15.7%
	shFEi	6.5%
CW9019	shFAi	14.1%
	shFEi	7.3%
Rh3	shFAi	26.9%
	shFEi	11.5%



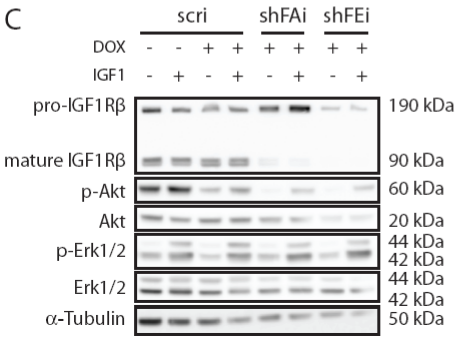


Figure S1. Validation of furin silencing in other cell lines and expression of other PCSKs. A) Furin silencing efficiency. Rh4, MRC5, CW9019 and Rh3 cells were treated with 25 ng/mL DOX for 48h, total mRNA was isolated and furin mRNA levels were studied by qRT-PCR. Data represent relative furin expression over non-treated scri control. B) Expression of other PCSKs upon furin silencing. Furin silencing was induced in Rh30 cells through addition of 25 ng/mL DOX, total mRNA was extracted 48h post induction and mRNA levels of other proprotein convertase family members were analysed by qRT-PCR. C) Rh4 cells were stimulated for 10 min. with 50 ng/mL IGF1 48h post induction of furin silencing and phosphorylation of IGF signaling pathway mediators Akt and Erk1/2 was examined by immunoblotting. One representative experiment is shown.

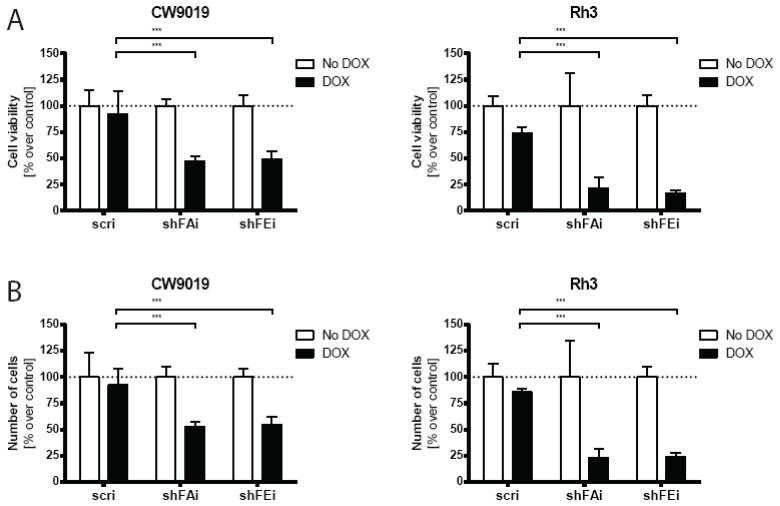


Figure S2. Cell viability in other aRMS cell lines is affected upon loss of furin activity. CW9019, Rh3 and MRC5 cells were treated with 25 ng/mL DOX for 96h. A) Metabolic activity was analysed in a

WST-1 assay. B) The number of cells was determined by staining with crystal violet. All data represent mean ±SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.005, two-way ANOVA.

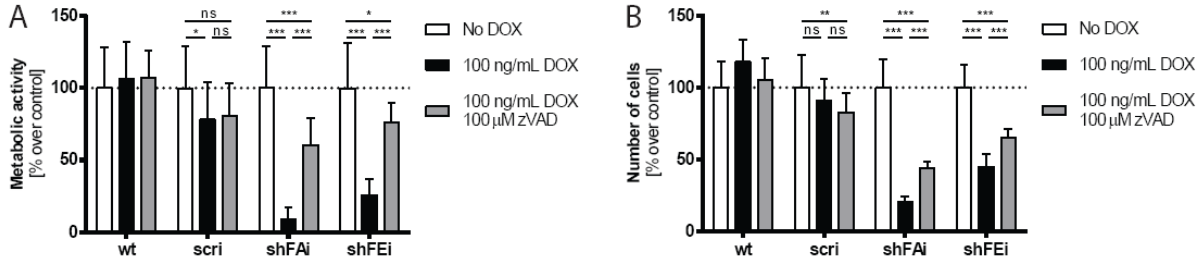


Figure S3. Caspase inhibition partially rescues cell viability in Rh4 cells. Rh4 cells were treated with 100 ng/mL DOX for 72h in the presence or absence of 100 µM zVAD. A) Metabolic activity was examined in a WST-1 assay. B) The number of cells was analysed by staining with crystal violet. Data represent mean ±SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.005, two-way ANOVA.

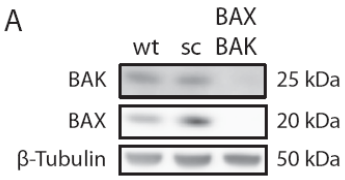


Figure S4. Validation of BAX/BAK knock out in Rh4 cells. Total protein of Rh4 wt, sc and BAX/BAK cells was extracted and BAX and BAK protein levels were analysed at protein level by immunoblotting.

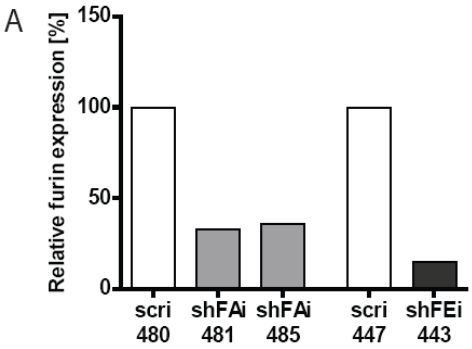


Figure S5. Furin silencing in Rh4 xenografts 5 days post induction. NOD/Scid mice were engrafted s.c. with 3.25 million Rh4 cells. Expression of shRNA was induced through administration of doxycycline (i.p. injection of 53 mg/kg doxycycline on two consecutive days combined with doxycycline supplemented food) once a tumour size of 250-300 mm^3 was reached. Mice were sacrificed 5 days post induction. Total mRNA was extracted from tumour tissue and furin mRNA levels analysed by qRT-PCR. Depicted are expression levels over HMBS.

Chapter 4

Discussion

1. Furin activity promotes survival and progression of rhabdomyosarcoma cells

In this work we investigated in depth the role of the proprotein convertase furin in the proliferative and malignant phenotype of RMS cells. We found that furin is a key regulator of the malignant phenotype of RMS cells *in vitro* and *in vivo*. Using diverse approaches to modulate furin activity, we could show that furin activity is required for cell growth and survival, and promotes angiogenesis as well as metastatic and invasive properties. All of these processes seem to be characterised by furin-mediated processing of cancer-related proproteins.

Lack of furin activity in RMS xenografts resulted in delayed tumour growth and was associated with decreased microvessel density, indicating that furin activity is required for angiogenesis. Reduced vascularisation of tumours upon furin inhibition has been confirmed by others who showed that ectopic expression of the inhibitor PDX or the prosegment of furin resulted in significantly decreased microvessel density in colon carcinoma, and head and neck carcinoma xenografts, respectively (Khatib, Siegfried et al. 2001; Lopez de Cicco, Bassi et al. 2005). As in our study, inhibition of furin led to impaired VEGF-C processing, suggesting that a lack of active VEGF-C prevents efficient neovascularisation in growing tumours. Additionally, initial reports suggested that hypoxia promotes HIF1-dependent enhanced expression of furin and relocalisation of furin from the TGN to the cell surface (McMahon, Grondin et al. 2005; Arsenault, Lucien et al. 2011), thus potentially facilitating processing of pro-angiogenic factors at the cell surface. However, whether this is also the case in RMS tumours remains elusive. Nevertheless, it is highly likely that impaired vessel formation, and thus reduced oxygen and nutrient supply, are responsible for delayed tumour growth in furin depleted RMS tumours.

Induction of furin silencing in aRMS cell lines significantly reduced proliferation of the cells. Moreover, half of the cell lines reacted in an even more sensitive way: depletion of furin in Rh3 and Rh4 cells induced caspase-dependent apoptosis *in vitro* and resulted in complete remission of Rh4 tumour *in vivo*. Apoptosis induction upon inhibition of furin through ectopic expression of the prosegment of furin was also seen in breast and colon carcinoma cell lines (Scamuffa, Sfaxi et al. 2014). Interestingly, the protein expression or activity levels of furin alone were not enough to predict the extent of the cellular response to furin silencing, as all four investigated aRMS cell lines showed similar levels of mature furin, and Rh3, Rh4 and Rh30 depicted comparable furin activity levels (see chapter 2, Figure 1). Intriguingly, a similar subclassification of sensitivity was reported for treatment of aRMS cells with an anti-IGF1R antibody (Cao, Yu et al. 2008). This suggests that the more pronounced dependency on furin might be coupled to IGF signalling levels and, potentially, to the downstream anti-apoptotic effects of the IGF-PI3K-Akt pathway, a hypothesis that warrants further investigations.

Unfortunately, the inducible furin silencing system could not be set up in eRMS cell lines due to insufficient expression of the tet repressor part of the construct, leading to leaky shRNA expression. Nevertheless, it would be of interest to determine whether a similar gradual sensitivity pattern can be detected in eRMS cells. To gain further insights on the sensitivity of eRMS and additional aRMS cell lines or, ideally, of patient derived cell lines, to inhibition of furin activity, the development and use of furin-specific inhibitors would be invaluable.

We further evaluated the impact of furin activity on the metastatic behaviour of RMS cells and found that furin activity promotes migration as well as invasion. Similar observations were made by Sfaxi et al. who investigated the effects of furin inhibition in colon carcinoma cells and confirmed decreased cell migration and invasion (Sfaxi, Scamuffa et al. 2014). The reduced metastatic potential correlated with reduced MMP2 and MMP9 activity upon ectopic expression of the PC inhibitor Spn4 and thus the authors hypothesised that PC inhibitors might be used for prevention of frequently occurring liver metastases of colorectal cancers (Sfaxi, Scamuffa et al. 2014). Furthermore, application of the pan-PC inhibitor PDX decreased the metastatic behaviour in lung adenocarcinoma and osteosarcoma cell lines (Liu, Li et al. 2014; Ma, Fan et al. 2014). Interestingly, opposing roles of distinct PCs in metastatic processes were also reported. Whereas specific inhibition of furin through ectopic expression of its prosegment decreased MMP9 activity and thus reduced the metastatic potential of breast cancer cells, inhibition of PACE4, also through prosegment expression, enhanced cell motility, migration and invasion in the same cells (Lapierre, Siegfried et al. 2007). This study shows the necessity of in depth investigations of distinct PCs in the tumour of interest and, eventually, the application of specific inhibitors in order to reduce the risk of unwanted side effects resulting from inhibition of other PCs.

2. The role of individual furin substrates

Many known cancer-related furin substrates are involved in one or more cellular functions that we could show to be altered upon modulation of furin activity in RMS cells, underscoring that furin promotes the malignant phenotype of RMS via processing of key precursor proteins. Deciphering the role of individual furin substrates is a challenging task, as mRNA and protein levels alone provide insufficient information. Thus, we decided to investigate the maturation status of some furin substrates previously shown to be involved in survival, growth, angiogenesis and metastasis of tumour cells.

IGF1R is a transcriptional target of the PAX3-FOXO1 fusion protein in aRMS (Ayalon, Glaser et al. 2001) and overexpression of IGF1R through gene amplification is frequently found in all RMS subtypes (Bridge, Liu et al. 2002). Furthermore, biallelic expression of the *IGF2* gene either through LOH or LOI (Zhan, Shapiro et al. 1994; Smith, Choufani et al. 2007)

results in overexpression of this growth factor, which stimulates autocrine IGF1R signalling in RMS cells (El-badry, Kohn et al. 1990; Minniti, Tsokos et al. 1994). Accordingly, increased levels of the different components of the IGF1R signalling pathway are associated with decreased survival of RMS patients (Blandford, Barr et al. 2006), suggesting the potential of targeting the IGF signalling pathway for treatment of RMS. To examine the importance of furin activity for functionality of this important growth factor pathway in RMS, we investigated IGF1R processing and found that active furin is required to mediate maturation of the receptor. Moreover, transient silencing of furin significantly impaired phosphorylation of the main downstream mediator Akt, highlighting the crucial role of furin activity for IGF signalling in RMS cells.

Similar to the IGF signalling axis, increased transcriptional levels of components of the PDGF signalling axis have been associated with negative outcome for RMS patients (Blandford, Barr et al. 2006). Hence, PDGFR-α has been proposed as a therapeutic target in RMS (Taniguchi, Nishijo et al. 2008). Whereas both PDGF-A and -B appear to undergo furin-like PC-dependent processing (Siegfried, Khatib et al. 2003; Siegfried, Basak et al. 2005), proteolytic cleavage of the other two ligands, PDGF-C and -D, seems to be dependent on the urokinase-type plasminogen activator (uPA) (Ustach and Kim 2005; Hurst, Najy et al. 2012). Thus, we examined PDGF-B maturation and could confirm that proteolytic processing of PDGF-B in RMS cells is indeed furin-dependent. In a recent study it was shown that PDGF signalling promotes cell proliferation, vessel density and macrophage infiltration in RMS tumours and likely regulates additional processes *in vitro*, including cancer cell stemness and differentiation (Ehnman, Missiaglia et al. 2013). That study was focussed on the more abundantly expressed PGDF-C and -D ligands, thus the role of PDGF-A and -B in RMS progression remains to be investigated.

VEGFs, belonging to the same family as PDGFs, are equally known to play a crucial role in tumour angiogenesis and lymphangiogenesis (reviewed in (McMahon 2000)). The two ligands VEGF-C and -D require processing by furin or related PCs to become fully active (Joukov, Sorsa et al. 1997; McColl, Paavonen et al. 2007). As we observed reduced microvessel density in RMS tumours with reduced furin activity, we investigated VEGF-C maturation and found that cleavage of pro-VEGF-C is dependent on furin activity. Whether furin activity has an equal impact on VEGF-D maturation and VEGF-D-dependent lymphangiogenesis in RMS tumours remains to be determined. Besides its pro-angiogenic function, VEGF signalling has been further implied in promoting tumour cell growth. Gee et al. investigated VEGF signalling in RMS cells and found evidence supporting the presence of an autocrine VEGF signalling axis promoting cell growth (Gee, Tsuchida et al. 2005). Whether

VEGF-C and -D posses a comparable potential of mediating cell growth remains to be investigated.

An additional furin substrate that was associated with angiogenesis is semaphorin-3B. In contrast to semaphorin-3F, which was found to act as an anti-angiogenic factor (Kessler, Shraga-Heled et al. 2004), the repellent effect of semaphorin-3B on endothelial cells appears to be kept low through furin-mediated cleavage and inactivation (Varshavsky, Kessler et al. 2008). Hence, overexpression of furin or furin-like PCs might promote neovascularisation of tumours through activation of angiogenic factors belonging to the VEGF/PDGF family or through inactivation of potentially anti-angiogenic factors like semaphorin-3B.

We also observed a reduced metastatic potential upon inhibition or silencing of furin, which was correlated with impaired maturation of the furin substrate MT1-MMP. Similarly, furin inhibition affected processing of MT1-MMP in osteosarcoma and lung adenocarcinoma cells and correlated with decreased migration and invasion (Liu, Li et al. 2014; Ma, Fan et al. 2014). Investigations of MMP expression patterns in RMS tumours have shown that the gelatinases MMP2 and MMP9, as well as the collagenase MMP1 are frequently overexpressed in aRMS tumours and were therefore hypothesised to contribute to the aggressive phenotype of aRMS (Diomedi-Camassei, Boldrini et al. 2004). MMP2 and MMP9 activity was reduced in breast cancer cells upon furin inhibition (Lapierre, Siegfried et al. 2007). Whether furin inhibition has a similar impact on MMP2 or MMP9 activity in RMS cells remains to be investigated. Nevertheless, matrix metalloproteases are furin substrates that are very likely involved in promoting the metastatic potential of RMS tumours.

3. Targeting furin activity for treatment of paediatric sarcomas

Our findings suggest that furin activity promotes malignancy of RMS cells by regulating the processing of cancer-related proproteins involved in growth, survival, angiogenesis and metastasis. Thus, we hypothesise that targeting furin activity will be of benefit for treatment of paediatric sarcomas. Since inhibition of furin will impair maturation of multiple furin substrates at once, we further predict that the application of furin inhibitors might be more potent than targeting individual furin substrates. For instance, targeting furin activity will not only abolish IGF1R maturation, but will also affect furin-mediated IGF1 and IGF2 processing (Duguay, Milewski et al. 1997; Duguay, Jin et al. 1998). Nonetheless, it might be advantageous to combine IGF1R and furin targeting strategies in order to increase the therapeutic benefit.

To our knowledge, the only furin-specific inhibitors that have been reported are dromedary heavy-chain-derived nanobodies, which were shown to bind and inhibit exclusively furin in a non-competitive manner (Zhu, Declercq et al. 2012). Unfortunately, these furin nanobodies have not been released for general use or clinical application, yet. All other

reported furin inhibitors are either not suitable for extracellular applications, e.g. the prosegment is a potent inhibitor, but has to be introduced through ectopic expression, or will also inhibit other PCs of the consecutive secretory pathway. Thus, the question arises of how important specificity towards one single PC is for a PC inhibitor. On the one hand, a broad acting PC inhibitor might display increased potency in inhibiting processing of substrates that can be cleaved by more than one PC. In contrast, potential side effects of unspecific PC inhibition in non-target tissue might be avoided through use of a specific furin inhibitor that more exclusively targets aberrantly high furin activity in RMS tumours. In the latter case redundancy in substrate processing through several PCs could even compensate for lack of furin activity in non-target tissue. In any case, tumour-targeted delivery of furin inhibitors will be the most promising approach to contain potential side effects caused by inhibition of furin activity in other tissues.

In order to estimate the therapeutic potential of furin targeting compounds several considerations should be taken into account prior to administration of the drug. Firstly, the endogenous activity of other furin-like PCs, possibly induced by furin inhibition, has to be investigated to ensure that furin-specific inhibition will not be compensate for by other PCs with redundant substrate processing function. Secondly, it will be crucial to decipher which furin substrates are the key mediators of cellular processes influenced by furin activity. Identification of key substrates might further serve to establish biomarkers in order to evaluate success chances of furin activity targeted therapy in RMS and other tumours.

Furthermore, we assessed expression levels of all PCs in other paediatric sarcomas and found that furin mRNA levels are, compared to the levels of other PCs, also elevated in osteosarcoma cell lines, but not in Ewing sarcoma cells. Liu et al. recently examined the potential of PC inhibition in osteosarcoma cells and found that application of the pan-PC inhibitor PDX diminishes the migration and invasion abilities (Liu, Li et al. 2014). Thus, furin and furin-like PC targeted therapy might be of benefit for prevention of osteosarcoma metastasis, a hypothesis that requires further investigation.

4. Immunomodulatory therapy

In very recent years Senzer et al. described for the first time the development of an autologous cancer cell vaccine that is partly based on silencing of furin (Senzer, Barve et al. 2012). This furin-knockdown and GM-CSF augmented (FANG) cancer vaccine is based on the genetic modification of patient-derived cancer cells to induce silencing of furin through expression of a bi-functional shRNA and expression of the granulocyte-macrophage colony-stimulating factor (GM-CSF), with subsequent reinjection into the patient to elicit an anti-tumoural immune response. Secretion of the cytokine GM-CSF leads to accumulation of

dendritic cells at the injection site, which in turn engulf and process injected cancer cells, resulting in tumour antigen presentation and mobilisation of tumour-specific T cells (reviewed in (Gupta and Emens 2010)). Simultaneous silencing of furin on the other hand helps to counteract immunosuppressive functions of TGFβ, which include the inhibition of cytolytic gene expression in cytotoxic T cells and natural killer cells (Rook, Kehrl et al. 1986; Thomas and Massague 2005). Since TGFβ members strictly require furin activity for proteolytic activation, a knockdown of furin eliminates potential TGFβ-mediated immunomodulatory properties, as adopted by many cancers to evade immune surveillance, and thereby enhances activity of the vaccine.

Safety of the FANG vaccine and efficacy of the immune response were confirmed and benefits of treatment were suggested by phase I trials in patients with advanced cancers including Ewing sarcoma and hepatocellular carcinoma (Nemunaitis, Barve et al. 2014; Ghisoli, Barve et al. 2015). Since TGFβ expression was confirmed previously in over 70% of RMS tumours and we found that most RMS tumours express furin (Wang, Guo et al. 2010), application of the FANG vaccine might represent a valuable new option for treatment of patients with RMS. Thus, investigations on the relation of TGFβ maturation and furin activity in RMS cells and tumours will be of further interest.

5. Furin-activated probes for imaging and therapy

Over the last decade, evidence has been gathered showing that cancer-related proteases, including MMPs and furin, may serve as powerful tools for conversion of protease-activatable imaging probes for visualisation and diagnosis of cancer, or for cancer-site specific activation of prodrugs.

Protease-activatable imaging probes are often composed of a fluorophore and quencher pair, thus quenching of the fluorophore in the native state keeps the molecule in an optically silent state, whereby liberation of the fluorophore from the corresponding quencher upon protease-mediated linker cleavage leads to fluorescent signal emission (reviewed in (Lee, Xie et al. 2010)). The fluorophores are chosen to operate preferably in the near-infrared spectrum, which improves tissue penetration and the signal-to-noise ratio (Weissleder and Ntziachristos 2003). The MMP-activatable near-infrared probe MMPsense 680, for instance, was applied to demonstrate the specificity of colorectal adenoma identification and was subsequently proposed for combined use with colonoscopy to improve early detection of colorectal lesions (Clapper, Hensley et al. 2011). As opposed to photochemical mechanisms, photoacoustic imaging based probes were described to provide even higher spatial resolution (reviewed in (Wang and Hu 2012)). In 2013, a first report emerged that described the design of furin-activatable photoacoustic probes and confirmed their specificity for furin activity-

dependent visualisation of breast cancer xenografts in mice (Dragulescu-Andrasi, Kothapalli et al. 2013).

Similar to imaging probes, protease-activatable prodrugs are inactive in their native state and undergo proteolytic cleavage of the inhibitory promoiety, converting the prodrug into its active form (reviewed in (Choi, Swierczewska et al. 2012)). Conversion of prodrugs is often dependent on overexpression of a certain protease in the target tissue, for instance enzymatically cleavable doxorubicin prodrugs were designed to be activated by uPA, a protease that is overexpressed in various cancers (Chung and Kratz 2006). Alternatively, selective cancer cell targeting may be achieved through coupling of the compound to a selective targeting agent such as an antibody recognising overexpressed cell surface receptors. These compounds are internalised upon receptor binding and undergo protease-dependent cleavage in the endosome, leading to subsequent release of the active drug into the cytosol. For instance, Wang et al. used HER2-targeted immunoproapototic proteins composed of a HER2 single chain antibody, a furin-cleavable linker and different human apoptotic molecules including caspase-3 and caspase-6 (Wang, Zhao et al. 2007). These immunoproapototic compounds are internalised and activated upon cleavage through endosomal furin and their antitumour activity was confirmed *in vivo* in HER2 positive tumours (Wang, Zhao et al. 2007).

Taken together, we hypothesise that the application of furin-activatable probes could be explored for non-invasive imaging of furin-positive tumours such as RMS in order to complement diagnostic modalities or to monitor success of therapy. Furthermore, prodrugs that are cleaved by cell surface-bound furin or compounds that undergo furin-dependent activation upon endocytosis represent promising new treatment strategies for RMS and other tumours with elevated furin activity.

6. Conclusion

In this work we have identified the PC furin as a novel key regulator of RMS malignancy *in vitro* and *in vivo*. We could show that furin activity plays an important role in diverse cellular processes, including cell proliferation, tumour growth, neovascularisation, metastasis and invasion, by regulating maturation of proproteins such as IGF1R, VEGF-C, PDGF-B and MT1-MMP. Furthermore, we uncovered a subgroup of aRMS cell lines, including Rh3 and Rh4, which are particularly dependent on furin activity and undergo apoptosis in the absence of functional furin, resulting in complete tumour remission *in vivo*. Since we could further confirm the presence of furin protein in 86% of the aRMS and eRMS tumour biopsies, we hypothesise that furin might be explored for novel targeted therapeutic approaches to treat children with RMS.

Importantly, distinct expression of furin in tumours offers several different therapy options. For instance, furin activity appears to promote many crucial cellular processes in RMS cells, suggesting that inhibition of furin through application of a specific furin inhibitor will improve the treatment of RMS patients. However, further investigations are required to examine the likely interplay of furin and the IGF signalling pathway in order to determine whether IGF1R levels are a biomarker to estimate responsiveness towards furin activity targeted therapy and to evaluate the potential of combined IGF1R and furin inhibition.

Furthermore, furin might serve as cell surface target receptor of RMS-homing peptides (Hajdin, D'Alessandro et al. 2010) or as protease to activate imaging probes or prodrugs in a tumour site specific manner. These approaches might be applied to improve early diagnosis of primary tumours or metastases detection, or to establish targeted delivery of drugs in order to decrease potential side effects due to action of chemotherapeutics in non-target tissue. Finally, a further therapeutic option is the application of autologous cancer cell vaccines like FANG, which already showed good results in Ewing sarcoma (Ghisoli, Barve et al. 2015).

In conclusion, we have demonstrated the importance of furin activity for RMS cells and predict that novel treatment options exploring either inhibition of furin activity or furin activity as a tool for tumour site specific delivery and activation of drugs and imaging probes might complement existing therapeutic options to improve diagnosis and treatment of paediatric RMS.

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